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Abstracts

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Contents

Analysis

Fully automated HPLC based analysis of cysteine and related compounds in plasma using on line microdialysis as sample preparation

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Low molecular weight thiols play important roles in metabolism and homeostasis. While plasma thiols, including metabolically related cysteine, glutathione, and homocysteine, are being investigated as potential indicators of health status and disease risk, trace levels, poor stability, and the lack of structural properties necessary for the production of signals compatible with common detection methods have hampered their accurate assessment. The facile oxidation of sulfhydryl compounds results in a variety of disulfide forms in vivo. To determine total plasma thiols – the sum of protein-bound forms, oxidized low-molecular-mass forms, and free reduced thiols – it is necessary to reduce disulfide bonds. In this presentation, a new procedure for fully automated assay of total cysteine, cysteinylglycine, glutathione, and homocysteine in human plasma is outlined. The oxidized and protein-bound thiols fractions are converted into their reduced forms employing sodium borohydride, and following derivatization with 2-chloro-1-methylquinolinium tetrafluoroborate and deproteinization by on line microdialysis, the S-quinolinium derivatives of analytes are separated, from each other and internal standard, and quantified by ion-pair reversed-phase HPLC with ultraviolet detection. The whole unattended instrument acquisition time is 13 min with no manual processing of sample. As a result about 80 samples a day can be assayed for total cysteine and related compounds. The method is linear within the normal and pathological ranges of thiols in humans and its sensitivity, precision and accuracy fulfils experimental and clinical requirements.

Determination of S-carboxymethyl-cysteine in cyclo-peptides and peptide-conjugates containing thioether bond

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Chemically stable thioether bond is frequently applied for the preparation of cyclopeptides and of peptide-conjugates possessing free SH group in one of the partner molecules. For the introduction of such bond the free thiol group of cysteine reacts with haloacetyl group attached to amino function of N-terminal amino acid or lysine side chain of the peptide. The homogeneity and the amino acid composition of new compounds was checked by amino acid analysis, analytical RP-HPLC and mass spectrometry. Qualitative and quantitative determination of S-carboxymethyl-cysteine (SMC) derived from this thioether linkage containing moiety was developed. Prior to amino acid analysis samples were hydrolysed in 6 N HCl in sealed and evacuated tubes at 110°C for 24, 36 or 48 hrs depending on the structure of molecules. The amino acid composition of the compounds was determined by two independent methods: using pre-column derivatization with o-phtaldialdehyde (OPA) reagent and post-column derivatization with ninhydrin. The appearance of SCM verified the success of cyclization and can be used for calculation of the degree of substitution in case of peptide-conjugates. The results of amino acid analysis were verified by the data of ES mass spectrometry method.

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Free and bound cysteine in homocystinuria: assessment of cysteine and glutathione status

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The raised concentration of protein bound homocysteine in homocystinuric (HCU) patients displaces protein bound cysteine and increases the free/bound cysteine ratio in plasma. This ratio is independent of albumin concentration. Results from 31 HCU patients were compared to 40 controls. Free cystine concentrations in HCU were poorly discriminated from the control range but the total cysteine results were almost invariably lower than control data. This appears to result from an increased free/bound cysteine ratio in HCU [mean (range) for control 0.50 (0.22–0.71) and for HCU 0.76 (0.32–1.75); $P = 0.0005$]. Ex vivo protein binding experiments in albumin solution revealed the free/bound cysteine ratio to be linearly related to the amount of homocysteine bound ($r = 0.907$, $P < 0.001$). We conclude that measurement of total cysteine is essential for assessment of the true cysteine status in HCU. However, any cysteine deficit, or alteration to free/bound cysteine ratios, does not obviously effect glutathione synthesis as assessed by measurement of plasma total glutathione.

Free D-amino acids in vertebrates

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In continuation of work on the screening of mammals for the occurrence of free D-amino acids (D-AAs), here we present data on quantities determined in urine and blood plasma of guinea pig, rabbit, goat, dromedary and cat, in the blood serum of fish, and in the brains of mole rat, rabbit, pig, bovine, seal, and rob. AAs were isolated by cation exchanger, converted into their *N*(*O*)-pentafluoro amino acid 2-propyl esters and quantified on a Chirasil-L-Val capillary column using mass spectrometric detection as described.

Representative data of animals investigated (n = 2–3) are shown in the Table. As can be seen free D-AAs were detected in the orders Rodentia (*Cavia aperea* f. *porcellus*, guinea pig); Lagomorpha (*Oryctolagus cuniculus* f. *domestica*, rabbit); Artiodactyla (*Capra aegagrus* f. *hircus*, goat); Artiodactyla (*Camelus dromedaries*, dromedary); Carnivora (*Felis libyca* f. *catus*, cat); and Osteichthyes (*Gadus morrhua*, codfish). Largest amounts of D-AAs were excreted with the urine. Free D-Ser (% relative to L-Ser) was detected in the brains (n = 1–2; cerebrum, wet weight) of mole (*Talpa europaea*, Insectivora)

Table 1. Quantities of D-amino acids (D-AA) in body fluids of vertebrates [% D = 100 D/(D + L)]

D-AA	guinea pig[a] urine µmol/L	%D	rabbit urine µmol/L	%D	serum µmol/L	%D	goat urine µmol/L	%D	plasma µmol/L	%D
Ala	452.9	61.9	50.2	53.5	3.4	1.3	3.7	5.2	0.5	1.0
Val	30.7	10.9	1.2	4.4	–	–	–	–	–	–
Thr	96.1	15.3	–	–	–	–	–	0.4	–	–
Pro	3.2	1.4	2.3	2.9	0.5	0.2	–	2.9	<0.1	0.1
Ser	99.5	27.2	245.4	69.8	2.5	0.9	2.5	12.6	0.2	0.9
Asx	17.9	11.8	31.5	30.2	1.1	1.1	4.0	16.5	0.1	2.3
Met	49.3	68.4	–	–	–	–	–	–	–	–
Phe	–	–	4.3	15.2	–	–	–	–	–	–
Glx	71.7	8.5	18.0	13.1	0.8	0.2	11.7	8.8	0.2	0.1
Tyr	–	–	16.9	26.8	–	–	–	–	–	–
Orn	89.7	33.7	9.0	19.5	0.6	0.7	0.2	3.0	–	–
Lys	0.9	9.7	7.3	2.5	0.8	0.7	0.2	0.6	0.7	1.7

D-AA	dromedary urine µmol/L	%D	serum µmol/L	%D	cat[a] urine µmol/L	%D	serum µmol/L	%D	codfish serum[b] nmol/L	%D
Ala	2.5	11.3	4.2	0.6	57.9	32.0	4.0	0.5	46	0.5
Val	–	–	–	–	7.4	9.9	–	–	–	–
Thr	–	–	–	–	4.2	1.4	–	–	–	–
Pro	0.5	1.9	–	–	0.9	4.4	1.5	0.8	12	0.4
Ser	2.9	7.9	–	–	159.9	61.6	2.0	0.8	–	–
Asx	3.9	14.8	1.8	1.2	18.4	50.9	1.0	2.0	27	4.3
Met	–	–	–	–	164.8	49.4	–	–	–	–
Phe	–	–	–	–	–	–	–	–	–	–
Glx	14.7	18.8	1.5	0.2	28.3	5.3	2.6	0.2	43	0.9
Tyr	–	–	–	–	–	1.5	–	–	–	–
Orn	1.9	5.8	–	–	12.6	32.5	–	–	8	1.7
Lys	0.5	1.2	1.4	0.7	61.4	23.0	1.2	0.4	41	0.7

(−) refers to not detected or not determinable; Asx = Asp + Asn; Glx = Glu + Gln; His, Arg, Trp, Cys not determined; [a] feed fortified with DL-Met; [b] nmol/g lyophilized serum.

(339 nmol/g; 21.6%); Sprague-Dawley rat (*Rattus norvegicus*, Rodentia) (n = 6; 98–381 nmol/g; 9.8–13.7%); rabbit (152 nmol/g; 11.3%); pig (*Sus scrofa* f. *domestica*, Artiodactyla) (221 nmol/g; 13.4%); bovine (*Bos primigenius* f. *taurus*, Artiodactyla) (284 nmol/g; 23.6%); seal (*Phoca vitulina*, Carnivora) (136 nmol/g; 3.8%), and rob (*Halichoerus grypus*, Carnivora) (218 nmol/g; 5.4%). From the date it is concluded that D-AAs are common in body fluids and certain tissues of vertebrates.

Characterization of total amino acid enantiomers in coffee

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Efforts have been made to characterize the two most common coffee varieties *arabica* and *robusta* using chemical data. The amino acids from coffee proteins represent an important fraction – about 11%. During roasting these compounds decompose and react, being responsible for the formation of several volatiles and browning compounds and, thus, for the character, and perhaps the acceptability, of the beverage.

The present study aimed to analyze the total D- and L-amino acids, in raw and roasted *arabica* and *robusta* coffees, from different geographical origins, in a total of 60 samples.

The hydrolysis method was extensively studied, in several time/temperature combinations, in order to avoid racemization. The classical HCl 6 M with and without phenol was tested as well as methylsulfonic acid 4 M, described as producing less racemization. Indeed, the last acid induced less formation of the D-aminoacids. The parameters choosed were 16 h at 110°C.

After hydrolysis the aminoacids were extracted with SCX columns, derivatized to their N-ethoxycarbonylethylesters and analysed by gas chromatography, with FID detection, on a Chirasil L-Val column (25 m).

Multivariate and nonparametric analysis applied to the chromatographic results proved to be a contribution in the *arabica/robusta* discrimination.

Determination of the enantiomers of methionine and cyst(e)ine in the form of methionine-sulphon and cysteic acid after performic acid oxidation by RP-HPLC

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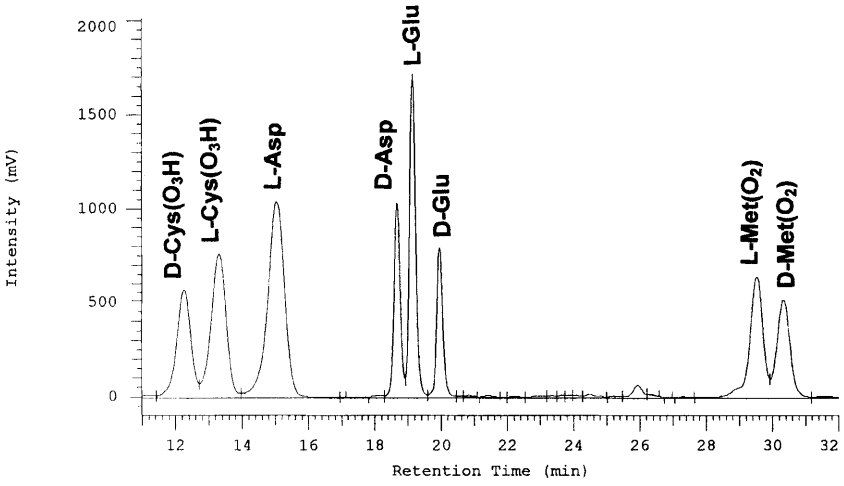
In order to determine the quantity of cyst(e)ine and methionine, the oxidation of cyst(e)ine and methionine



into cysteic acid and methionine sulphone with performic acid is often applied before hydrolysis of protein. The authors examined the applicability of this process in case of quantification of cyst(e)ine and methionine enantiomers. The RP-HPLC analytical method was developed for the determination of the amount of cysteic acid and methionine sulphone enantiomers. The rate of conversion during oxidation from cyst(e)ine into cystic acid and from methionine into methionine sulphone was determined. The racemisation of L-cyst(e)ine and L-methionine was negligible during oxidation with performic acid, therefore this process can be applied before hydrolysis during quantification of cyst(e)ine and methionine enantiomers. After the performic acid oxidation and the 6M HCl hydrolysis of the protein, OPA/TATG (o-phthaldialdehyde/tetra-O-acetyl-1-thio-β-D-glucopiranoside) precolumn derivatisation method was used, and the enantiomers of sulphur containing amino acids were separated by RP-HPLC (LiChrosphere 100 RP-18e, 125 × 4mm, 5μm column, Merck-Hitachi LaChrom HPLC). The resolution of the peak of cysteic acid and methionine sulphone enantiomers was better than 1,5. The method was used to determine the amount of L- and D-cyst(e)ine and L- and D-methionine containing preparations prepared by fermentation and subsequent purification. The separation of the enantiomers of cysteic acid, methionine sulphone, aspartic acid and glutamic acid is displayed on the chromatogram.

The introduction of amino acid racemisation based age estimation into paleoanthropological research

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This study describes the application and results of an age estimation method so far not introduced into the inventory of historical anthropology. The teeth of 126 individuals (age range 17–86 years) were analysed by the authors, and high degree of correlation was found between the D-aspartic acid content, the

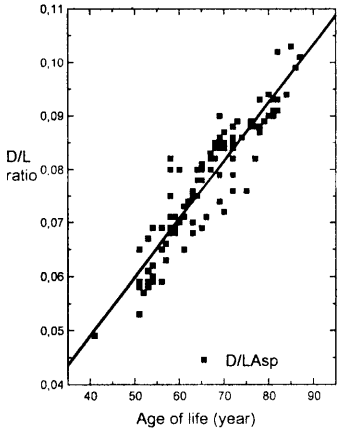

Fig. 1. Linear regression between the age of life of the individuals and the D/L aspartic acid ratio of their teeth

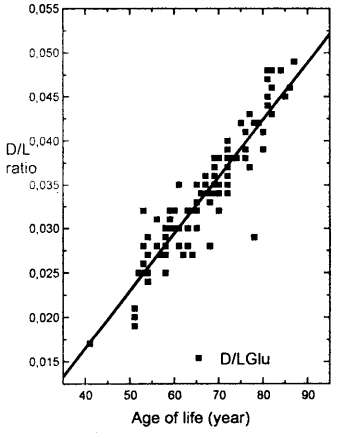

Fig. 2. Linear regression between the age of life of the individuals and the D/L glutamic acid ratio of their teeth

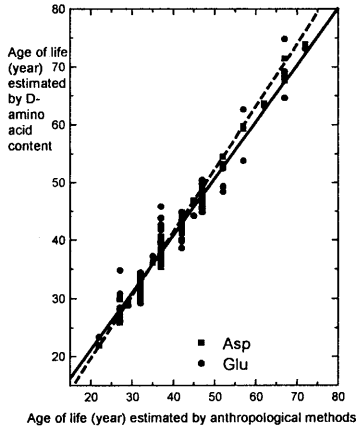

Fig. 3. Linear regression between the age of life estimated by anthropological methods and D-amino acid content

D/L rate of aspartic acid and the individual age of specimens. A method for age determination based on D-aspartic acid content and on the racemisation of L-aspartic acid of teeth was developed. D-glutamic acid, beside D-aspartic acid, was found to be eminently suitable for the estimation of individual age, as it showed a sufficiently high sensitivity. Calibration curves based on these investigations were used for the age estimation of 65 adults (39 males and 26 females) of unknown individual age from the Avar Period series of Kereki-Homokbánya (Hungary). The age distribution of the sample was the following: 39 individuals (60%) belonged to the adult age group, 22 persons (34%) to the mature and 4 (6%) to the senile one. The correlation between our results and those obtained using standard paleoanthropological methods was over 0.9.

Quantitative determination of free and bound 3-nitrotyrosine in rat plasma and tissues using isotope dilution liquid chromatography-electrospray tandem mass spectrometry

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Since 3-nitrotyrosine was reported to be readily formed in proteins by reactions with nitrite or nitrogen dioxide, it has been postulated to be a possible marker for investigating peroxynitrite-mediated nitration of proteins. Thus, several methods were developed to assess nitration of tyrosine in proteins and determine 3-nitrotyrosine in physiological fluids. Methods based on HPLC or GC/MS techniques were described to quantify 3-nitrotyrosine within tissues or biological fluids. Unfortunately, it has been demonstrated that an artifactual nitration of tyrosine occurs with GC/MS assays leading to an overestimation of the response. In the present work, LC-ESI-MS/MS methods for quantification of free 3-nitrotyrosine in rat plasma as well as bound 3-nitrotyrosine in tissue samples are reported.

Plasma samples were spiked with 2,5,6-d_3-3-nitrotyrosine and the following steps were applied prior to injection into the LC-ESI-MS/MS system used in selected reaction monitoring (SRM) mode (m/z 283 → 181 for the analyte and m/z 286 → 184 for the internal standard): protein precipitation, solid phase extraction on aminopropyl cartridge and derivatization in *n*-butanol in HCl 3 N. 3-Nitrotyrosine butyl ester has lead to a dramatic increase of the sensitivity (*ca.* 5-fold) by comparison with 3-nitrotyrosine. Under such conditions, calibration curves exhibited excellent linearity ($r^2 > 0.99$) within concentration range 0.3 to 28.5 nM (equivalent to 47.3–4,730 fmol on column) and recoveries above 85%. Inter- and intra-assay precision was determined below 15% over the concentration range 1.4 to 28.5 nM. No artifactual nitration of tyrosine occurring during sample clean-up was observed. This was unambiguously established by plotting experimental ratio of analyte response/internal standard response versus expected within the range 0.3–28.5 nM. This curve strongly correlated with a linear model ($r^2 > 0.99$) and slope was 1.07 ± 0.06 (mean ± SD). Basal level of 3-nitrotyrosine in rat plasma was measured to be within concentration range < LOD to 1.5 nM.

3-Nitrotyrosine basal level in rat plasma, kidney and liver proteins was established by performing enzymatic hydrolysis in order to avoid artifactual nitration of tyrosine which may occur under strong acidic conditions (HCl 6 N at 120°C). Resulting hydrolysates were analysed by LC-ESI-MS/MS and 3-nitrotyrosine was monitored in SRM mode (m/z 227 → 181 for the analyte and m/z 230 → 184 for the internal standard).

Zinc binding to SET protein – A capillary electrophoresis study

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The *set-can* fusion gene was first detected as associated with acute undifferentiated leukemia. SET (also called PHAP II) is a nuclear phosphoprotein with a long acidic tail. SET has been shown to inhibit phosphatase PP2A and is a substrate of human granzyme A. In order to determine any Zn(II) binding properties of SET, we utilized affinity capillary electrophoresis (ACE) to detect shifts in mobility as Zn(II) ions bind to the protein. We have earlier employed ACE to measure the binding constants of Zn(II) to the nucleocapsid protein of HIV-1. With a constant concentration of recombinant SET as a receptor and varying concentrations of Zn(II) as ligand in the sample buffer, we observed changes in electrophoretic mobilities of SET when complexes were formed with Zn(II). Scatchard analysis of the mobility provided the stoichiometry and binding constant of Zn(II) to SET.

Peptaibol antibiotics trichoaureocins from the mold *Trichoderma aureoviride*

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Peptaibols are defined as fungal polypeptides containing a high proportion of Aib (α-aminoisobutyric acid) and a *C*-terminal bound amino acohol. The mold *Trichoderma aureoviride* (strain IMI 91968; Commonwealth Mycological Institute, Kew, UK) was cultured in complex medium consisting of casein peptone, 17 g; soy peptone, 3 g; yeast extract, 5 g; D-glucose, 2.5 g; NaCl, 5 g; dipotassium hydrogen phosphate, 2.5 g in 1 L demineralized water adjusted to pH 6.8. Fermentation was conducted in nineteen 2-L shake flasks, each containing 400 ml medium, for 7 d at 27°C. Mycelia were obtained by filtration and extracted with MeOH and MeOH/chloroform. Extracts were evaporated to dryness and subjected to Sephadex

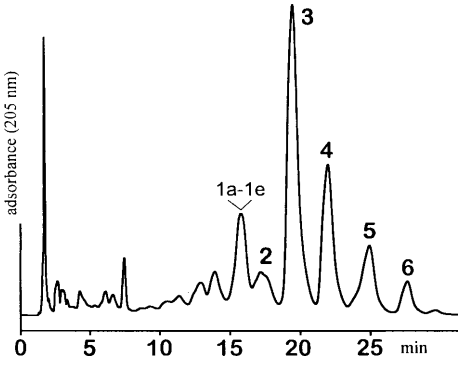

Fig. 1. Analytical HPLC of the microheterogeneous mixture of trichoaureocins; for correlation of peak numbers and sequences see Fig. 2; Chromatography: Supersphere 100 RP-18 (Merck), 250 mm × 4 mm ID, 4 μm; gradient elution (A) MeCN/water/MeOH 39/22/39, (B) MeCN/MeOH (1:1); peak no. 1 consists of five peptaibols (nos. 1a–e) resolvable on a fluorocarbon HPLC column

No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1a	Ac	A	A	U	A	U	A	Q	U	V	U	G	L	U	P	V	U	V	Q	Q	Fol
1b	Ac	U	A	U	A	A	A	Q	U	V	U	G	L	U	P	V	U	U	Q	Q	Fol
1c	Ac	U	A	U	A	U	A	Q	U	V	U	G	L	U	P	V	U	A	Q	Q	Fol
1d	Ac	U	A	U	A	U	A	Q	U	V	A	G	L	U	P	V	U	U	Q	Q	Fol
1e	Ac	U	A	U	A	U	A	Q	A	V	A	G	L	U	P	V	U	U	Q	Q	Fol
3	Ac	U	A	U	A	U	A	Q	U	V	U	G	L	U	P	V	U	U	Q	Q	Fol
4	Ac	U	A	U	A	U	A	Q	U	X	U	G	L	U	P	X	U	X	Q	Q	Fol
5	Ac	U	A	U	A	U	U	Q	U	V	U	G	L	U	P	V	U	U	Q	Q	Fol
6	Ac	U	A	U	A	U	U	Q	U	X	U	G	L	U	P	X	U	X	Q	Q	Fol

Fig. 2. Sequences of trichoaureocins; *Ac* acetyl; protein amino acids are abbreviated according to one-letter-nomenclature; *U* Aib, *X* valin (*V*) or isovaline (*Iva*); *Fol* phenylalaninol; Fol and chiral amino acids are of the L-configuration with the exception of D-Iva

LH-20 (eluent MeOH) and silica gel chromatography (eluent chloroform/MeOH/AcOH/water 65:25:3:4) yielding 2.83 g and 0.9 g, respectively, crude peptaibol mixture named trichoaureocins. The peptide mixture was uniform on TLC but could be separated by analytical (Fig. 1) and semipreparative HPLC (Nucleosil 100 C-18; 250 × 8 mm ID; 3 μm). Six peptides could be isolated each of which was subjected to sequencing using on-line HPLC (fluorocarbon stationary phase) ESI-MS/MS (LCQ, ThermoQuest, Finnigan MAT) as described for peptaibols trichovirins and antiamoebins. Sequences are presented in Fig. 2. The 20-residue peptaibols represent a natural peptide library and cause hemolysis of sheep erythrocytes and exert antibiotic activity against *Bacillus subtilis* and *Staphylococcus aureus*.

Determination of amino acids in wine by one- and two-dimensional NMR methods

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Wine consists of several hundred components present at different concentrations. The dominant ones are water, ethanol, glycerol, sugars, organic acids, and various ions, while amino acids are present at much lower concentration. The composition of amino acids is of great importance in wine production. They act as a source of nitrogen for yeast during fermentation, they influence the aromatic composition of wine and their composition can be used to differentiate wines according to vine variety, geographical origin, and year of production.

Among already established analytical methods high-field NMR has been shown to be a promising method for the non-destructive analysis of low-molecular mass compounds in complex mixtures like wine due to its selectivity and capability of simultaneously detecting a great number of compounds. ^{1}H and ^{13}C one-dimensional NMR spectra of wine are very crowded and many signals are overlapped. Due to a great difference in concentration levels the signal intensities of particular compounds may vary for the factor of 25. The tails of the dominant frequencies of water, ethanol and glycerol obscure weak signals of minor compounds like amino acids in the near surroundings. The use of 2D homo- and heteronuclear experiments and the suppression of strong signals are a prerequisite for a successful ^{1}H and ^{13}C signal assignment. A complete assignment of ^{1}H and ^{13}C NMR resonances of seventeen amino acids commonly present in wine and of γ-aminobutyric acid at pH 3 was accomplished using gradient-selected COSY, TOCSY, gradient-selected HSQC and HMQC experiments with incorporated WET pulse sequence for the supression of large signals.

Unambiguous assignment of ^{1}H and ^{13}C NMR resonances of amino acids is necessary for the selection of appropriate signals in fast and simple one-dimensional NMR that can serve as parameters in the chemometric classification of wines according to the provenance, vine variety, and year of production.

New method of determination of tryptophan in peptides and proteins

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Highly sensitive colorimetric method for determination of aldehydes in the reaction with N-methyl benzothiazolone hydrazone (MBTH) turned out to be not very specific for such carbonyl compounds. Namely, it has been found that tryptophan and to higher degree its N-derivatives (N-acetyl-Trp, Ala-Trp, Gly-Trp) and also tripeptides (Gly-Trp-Gly and Leu-Trp-Leu) in the reaction with MBTH and Fe^{3+} are converted to coloured products, with maximum wavelength at 595 nm. The properties of the products and the kinetics of the reaction under defined conditions are described in the spectrophotometric procedure.

Proteins containing tryptophan are also substrates in the reaction with MBTH. Comparison of molar extinction coefficients of MBTH-Fe^{3+} – treated various proteins with those of simple N-derivatives of tryptophan shows, that not all molecules of tryptophan in proteins are accessible to the reagents, and in order to determine all tryptophan moieties partial unfolding of protein has to be performed.

It should be emphasized that aldehydes cannot be detected and accurately determined in the presence of tryptophan derivatives and protein, and also aldehydes interfere with determination of tryptophan derivatives.

A reagent for the detection of protein amino acids

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Detection of protein amino is of utmost importance for the evaluation of protein structure and also their presence in numerous natural products. Several specific and non-specific reagents have been used for their detection using thin-layer chromatography, an important tool for such purpose. Of the reagents in general use, ninhydrin is the most popular for its high sensibility, however, nihydrin produces same purple color with most of the amino acids (only proline and hydroxproline produce yellow color). An endeavour has been made to resolve this color problem with a reagent which is capable of developing various distinguishable colors with many of the protein amino acids and also shows its high sensitivity comparable to ninhydrin. A probable mechanism for such color formation has also been proposed.

Analysis of stable nitrogen and carbon isotope enrichment in amino acids by GC-C-IRMS

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Gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) is a highly sensitive approach to analyze amino acid $^{13}C/^{12}C$ and $^{15}N/^{14}N$ isotopic compositions in the range of natural isotopic abundance or in slightly enriched samples. Since only small amounts of material are required this technique becomes particularly valuable in tracer experiments

measuring enrichments below the sensitivity range of conventional GC-MS. The GC-C-IRMS technique combines the resolution capabilities of GC with the accuracy and precision of IRMS. At low abundance GC-C-IRMS analysis it is superior in terms of time, labor, and sample requirement as compared to the conventional off-line analysis. We discuss some latest advancements and applications of GC-C-IRMS amino acid analysis related to nutrition research. Plasma amino acids in omnivorous human subjects show a characteristic ^{15}N-isotopic pattern with phenylalanine and threonine showing the lowest abundance, whereas e.g. alanine and leucine are higher by 25‰ $\delta^{15}N$. In rats fed diets containing intrinsically labeled ^{13}C casein or the corresponding amino acid mixture labeled with ^{13}C leucine and ^{15}N lysine whole-body protein homeostasis is better supported by casein-bound than free amino acids. There is no adaptation to a low lysine diet by an enhanced bioavailability of intestinal microbial lysine to extra-splanchnic tissues in minipigs.

Highly selective HPLC determination of tyrosine, tryptophan and their related compounds based on precolumn derivatization followed by intramolecular fluorescence resonance energy transfer detection

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We have developed highly selective HPLC method for the determination of tyrosine, tryptophan and their related compounds (L-DOPA, catecholamines, 5-hydroxytryptamine, etc.). The compounds were precolumn-derivatized with a commercially available fluorogenic reagent for amines by usual manner. Each derivative afforded intramolecular fluorescence resonance energy transfer (FRET) from the tyrosyl or tryptophoryl moiety (donor) to the labeled fluorophore (acceptor); the acceptor fluorescence was observed with the excitation of the donor at 280 nm. The derivatives were separated on a reversed-phase column and then effectively detected by monitoring their FRET. Through the screening study of 11 fluorogenic reagents, o-phthalaldehyde (with 2-mercaptoethanol) and Dansyl chloride gave the best results for the purpose. The FRET detection method was highly selective and sensitive by comparison with the previous methods detecting native fluorescence of the compounds or typical fluorescence of the acceptor.

Calorimetric studies of interactions between *N*-acetylamino acid amides and urea in water at 298.15 K

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The presented study was devoted to determination of the energetic effect of interactions in aqueous solutions between urea and neutral amino acid derivatives. The principal reason for studying of interactions of peptides with urea is the hope that such investigations will give insight into the factors affecting protein denaturation in aqueous solutions.

The enthalpies of solution of *N*-acetylglycinamide, *N*-acetyl-L-alaninamide and *N*-acetyl-L-leucinamide were measured in water and in aqueous solutions of urea of molality 0.25 to 3.0 $mol \cdot kg^{-1}$ using the "isoperibol" type calorimeter at 298.15 K.

From the obtained standard dissolution enthalpies $\Delta_{sol}H_m^{\infty}$ the enthalpic pair interaction coefficients h_{xy} for urea-*N*-acetylamino acid amide pairs in water were calculated. These parameters derived from McMillan-Mayer theory are regarded as a measure of effect of interactions between solute molecules in solution.

The h_{xy} values for the systems investigated suggest that the interactions between urea and amide molecules dominate the effects of dehydration of nonelectrolyte and of peptides. The replacement of the hydrogen atom in the hydrocarbon chain with a methyl group causes a positive change in the value of the enthalpic pair interaction coefficient.

The obtained results were compared with those of earlier studies of interactions between electrolytes, namely sodium chloride, potassium chloride and sodium iodide and the same *N*-acetylamino acid amides. The effect of the solute type on the magnitude of the interaction parameter was also analysed.

The enthalpic pair interaction coefficients of L-α-amino acids as a hydrophobicity parameter of amino acid side chains

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The side chains of amino acids in solution react in various ways with the water molecules which surround them as well as with other components of solution depending on the fact whether they possess non-polar, polar or ionic groups. Many research laboratories carry out studies intended to describe precisely the intermolecular interactions with the participation of amino acid side chains.

Such a description may allow one to describe better the spatial structures of protein and the mechanisms of folding its surface area.

The present work reports the results of calorimetric measurements of the dilution enthalpies of L-α-amino acids in water. Using modified McMillan-Mayer's theory, these results served to calculate the enthalpic homogeneous interaction coefficients which characterise interactions between the amino acid zwitterions with the competitive participation of water molecules. Thus, these coefficients illustrate the differences in amino acid molecules interactions both with the homogeneous amino acid molecules and water molecules around them, and consequently they may play the part of a parameter which differentiates the hydrophobic/hydrophilic properties of amino acid side chains.

The enthalpic interaction coefficients of the homogeneous pairs of L-α-amino acids were compared also with the hydrophobicity parameters obtained by Fauchere et al., which describe the side substituents of natural amino acids as well as aminobutiric acid (Aba).

Based on the above statement, one may conclude that the obtained enthalpic homogeneous pair interaction coefficients of L-α-amino acids in water make it possible to systematise amino acid side chains according to their affinity to water or their hydrophobic-hydrophilic properties. Thus the enthalpic homogeneous pair interaction coefficients may play the role of parameter describing the lipophilicity (hydrophobicity) of amino acid side chains.

X-ray investigation of the structures of cluster rhenium compounds with amino acids

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Recently we have reported about synthesis and some chemical and biological properties of a range of cluster rhenium

compounds (III) with amino acid ligands. In this work we present results of X-ray investigation of fourth amino acid complexes of rhenium (III), which have different coordination of amino acids around binuclear complexforming center – Re^{6+}. Substances $(GlyH)_4[Re_2Cl_8]Cl_2$, $(GlyH)_2[Re_2Cl_8]\cdot H_2O$ and $(\beta\text{-}AlaH)_2[Re_2Cl_8]$ have amino acid ligand in the external sphere of coordinating center RE_2^{6+} and substance $[Re_2(GABA)_2Cl_5(H_2O)]Cl\cdot 2H_2O$ – in inner, but GABA has cis-position according to Re – Re bond. Influences of fatty radical length in the amino acid ligand on week interaction between binuclear anion $[Re_2Cl_8]^{2-}$ and protonized amino acid are discussed. Role of hydrogen bonds in formation of crystal unit cell of investigated substances is shown. These two factors are the reason of formation of staggered conformation of an anion $[Re_2Cl_8]^{2-}$ in the substance $(GlyH)_4[Re_2Cl_8]Cl_2$ together with existence of quadruple Re – Re bond that is described first.

In the substance $[Re_2(GABA)_2Cl_5(H_2O)]Cl\cdot 2H_2O$ axial position of Re_2^{6+} fragment are substituted by ligands of different kind: H_2O and Cl^- – that says about possibilities to coordinate a substrate of biological nature exactly to these position.

Separation and quantification of α, β and κ caseins in ewe, goat and cow's milk by *HPLC/UV*

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A precise, sensitive and reliable RP-HPLC/UV method was developed to enable determination of α, β and κ caseins in cow's milk. The optimised method using a Chrompack P-300-RP column allowed separation of caseins in 30 min. This column differs from conventional alkyl-bonded silica RP matrices in that it is an underivatised polystyrene-divinylbenzene matrix, a material which proved excellent chemical and pH stability. Gradient elution was carried out at a flow rate of 1 ml/min and a temperature of 46°C, using a mixture of two solvents. Solvent A 0.1% trifluoroacetic acid in water and solvent B was 95% acetonitrile-5% water-0.1% trifluoroacetic acid. The effluent was monitored by a UV detector at 280 nm.

The determinations were performed in the linear range of 0.038–0.38 mg/ml for κ-casein, 0.19–1.9 mg/ml for α-casein and 0.15–1.5 mg/ml for β-casein. The detection limits were 0.037, 0.03 and 0.0075 mg/ml, respectively. The validity of the method was verified. The recoveries ranged from 91 to 100% for cow's milk. The precision of the method was also evaluated, the % CV being less then 3.67%.

The developed methodology was also applied with success to the separation of caseins in ewe and goat milks. Different chromatographic profiles were obtained for the three kinds of milk.

Study on the free nonprotein amino acids and N-γ-glutamyl oligopeptides of *Panax* species

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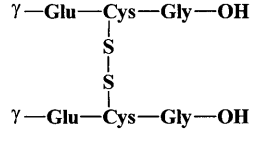
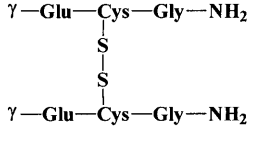
Previous Studies of nonprotein amino acids and oligopeptides in *Panax* species including *Panax* ginseng, *Panax* notoginseng etc. were reviewed. γ-amino butyric acid (GABA), neuroexcitotoxic nonprotein amino acid, β-N-oxalo-L-α,β-diaminopropionic acid (β-N-L-ODAP) and its α-isomer, β-Alanine, L-citrulline, L-ornithine as well as L-α-aminoadipic acid were isolated and identified from *Panax* species. Six γ-glutamyl oligopeptides were isolated for the first time from aqueous methanol extracts of *Panax* ginseng root. Their structures had been established, as P-I (oxidized glutathione, GSSG) and its reduced form, P-II (oxidized glutathione amide), P-III (N-γ-glutamylglycylcysteine disulfide, IGSSG), P-IV (N-γ-glutamylcystinyl-bis-glycine), P-V (N,N′-bis-γ-glutamylcystinylglycine) and P-VI (γ-glutamylarginine). Five of them are related to oxidized glutathione. The structures were further confirmed by the chemical syntheses. P-III is apparently a new biologically active peptide which exhibits somnogenic effects. It is more potent than P-I. The neurophysiological activity of β-N-L-ODAP and GABA as well as other bioactivities are also discussed.



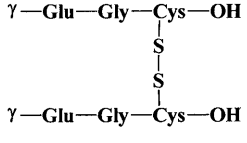
P-I

oxidized glutathione (GSSG)



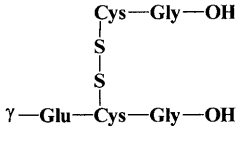
P-II

oxidized glutathione amide



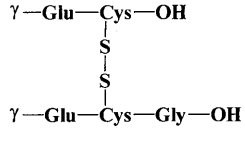
P-III

N-γ-glutamylglycylcysteine disulfide (IGSSG)



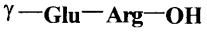
P-IV

N-γ-glutamylcystinyl-bis-glycine



P-V

N,N'-bis-γ-glutamylcystinylglycine



P-VI

γ-glutamylarginine

Biology

Physiological roles of free D- and L-alanine in invertebrates

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Several aquatic crustaceans and bivalve molluscs accumulate a large amount of free D-alanine (3–50 μmol/g wet wt.) in their muscle tissues. During seawater acclimation from freshwater to 75% seawater, red swamp crayfish *Procambarus clarkii* largely accumulated D- and L-alanine by 6.8- and 4.5-fold, respectively, together with L-glutamine, L-proline, and glycine. The percentage of D-alanine to total alanine increased from 38% in freshwater to 48% in 75% seawater. These data indicate that D- and L-alanine are the major compatible osmolytes responsible for the intracellular isosmotic regulation of this species as well as other crustaceans.

Under anoxia stress for 12 h in freshwater, 50 and 75% seawater, crayfish increased D- and L-alanine in muscle and hepatopancreas in addition to the increase of lactate. The increase was much higher in seawater than in freshwater. Thus, D- and L-alanine may be anaerobic end products during prolonged anoxia of this species.

Alanine racemase [EC 5.1.1.1] has been proved to catalyze the interconversion of D- and L-alanine in crustaceans and bivalve molluscs. This enzyme was isolated to homogeneity from the muscle of black tiger prawn *Penaeus monodon*. The purification was 127,600-fold with 16% yield. The molecular weight of the enzyme was estimated to be 45 kDa on SDS-PAGE and 90 kDa on gel filtration, suggesting the dimeric nature of this enzyme. The amino acid sequences of the peptide fragments obtained from the isolated enzyme showed low homology below 50% with those of microbial enzymes.

Syntheses and immunological effect of thymic humoral factor-γ2 analogues

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Nine analogues of thymic humoral factor (THF)-γ2, were prepared by the solid-phase method and their in vitro restoring effect on the impaired blastogenic response of phytohemagglutinin (PHA)-stimulated T-lymphocytes of uremic patients with infectious diseases were examined. The results were as follows: [Arg6]-THF-γ2 exhibited higher restoring activity than that of our synthetic THF-γ2. [Sar4]-, [Val1]-, [Arg3]-, [Gly5]-, and [Asn3]-THF-γ2 were also active, but less potent than that of our synthetic THF-γ2. Other three peptides, [βAla4]-, [Arg2]-, and [Gln2]-THF-γ2, did not show any restoring activity on the impaired blastogenic response of uremic patients with infectious diseases.

The use of covalent mRNA-peptide fusion technology in the directed evolution of peptides and proteins

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Phylos has developed a powerful combinatorial biology platform for peptide and protein selections. Phylos' proprietary PROfusion™ technology enables the selection of peptides and proteins with desired properties. The fundamental advance represented in this unique platform is the *in vitro* covalent linkage of a peptide or protein (phenotype) to the encoding messenger RNA (genotype). This linkage permits the selection of a protein based on its characteristics and allows the recovery and amplification of that protein through PCR, an efficient means of bring the desired proteins to easily detectable levels. PROfusion™ technology has routinely selected peptide and protein binders with affinity constants in the nanomolar to picomolar range. The starting library size of randomized peptide or protein PROfusion™ constructs is typically 10^{13}. Linear and constrained loop peptide libraries, for ligand generation, enzyme: substrate interaction, peptidomimetic design, and epitope mapping have been successfully used. Randomized constrained loops have also been incorporated in a beta-sandwich scaffold, resulting in the successful selection of binders against targets of therapeutic interest.

Epitope mapping of *de novo* proteins with fragments from human interferon-α2 and insulin

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Antigenic properties of three biological active *de novo* proteins were investigated by peptide scanning approach, using noncleavable Multipin technology. A *de novo* protein albebetin (PID CAA47376) was engineered to attain a pre-designed 3D structure and later modified by grafting short peptide fragments from human α_2 interferon (AAG59605), and insulin molecules (AAG59606). Such protein constructs carrying important biological activities may be used in future as potential protein pharmaceuticals. Despite artificial proteins are investigated for more than 10 years, immunological properties of these substances are not known. In our experiments we applied an innovative approach of raising antibodies in yolks of egg-laying hens. Three continuous antigenic determinants with different immunogenic potentials have been revealed in two proteins with partially overlapping sequences. It was shown that the octapeptide interferon fragment is the immunodominant site in albeferon and albeferon-insulin molecules. On the contrary, the hexapeptides, corresponding to the insulin fragment displayed low immunogenic activity. Thus we recognise that the fragments attached to the *de novo* frame could essentially govern immunological properties of resulting construct.

No preference of any type of secondary structure was observed in antigenic determinants. Nevertheless, all of them are located at the boundaries of the secondary structure elements and on the predicted surface-located sites of albebetin molecule.

Study of biological properties of de novo proteins inclusive active fragments from human α_2-interferon and insulin

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[2] Gamaleya Research Institute of Epidemiology and Microbiology, Moscow, and
[3] Institute of Biomedical Chemistry, Moscow, Russia

Peptide fragments from human α_2-interferon and insulin corresponding to the functionally important sites of their molecules were grafted into *de novo* protein albebetin (PID CAA47376) engineered to attain a pre-designed tertiary structure with a unique topology that has not been observed in natural proteins. By means of genetic engineering the DNA fragments corresponding to these peptides were inserted into the albebetin gene to obtain two variants of albebetin with antiviral fragment of human α_2-interferon and two variants of albebetin with insulin-like peptide. The chimerical genes were expressed in *Escherichia coli* in a fusion expression system with thioredoxin based on the plasmid *pET-32* (Novagen). The fusion proteins were digested by highly specific protease factor *Xa* and the target chimerical proteins were purified and tested for their structure and biological activity.

According to the CD spectroscopy study the chimerical proteins maintained the pre-designed structural properties of albebetin. Toxicological testing of the proteins in the MTT-test did not reveal their cytotoxicity. Antiviral activity of *de novo* proteins with human α_2-interferon fragments was studied *in vitro* using human fibroblasts cell line *L-41* and simian cell line *VERO*. Treatment of these cell lines with the proteins revealed the dose-dependent stimulated antiviral activity on fibroblasts and direct dose-dependent antiviral activity on the *VERO* cells. One of two *de novo* proteins including insulin-like fragment (PID AAG59607) acquired ability to stimulate glucose uptake by *L-929* cells although the efficacy of stimulation was lower than that for the synthetic peptide and insulin. These results demonstrated that albebetin can be used as a scaffold for constructing of the functionally active *de novo* proteins possessing the pre-designed tertiary fold of albebetin and various biological activities.

Identification of TAA peptides for anti-tumor vaccines

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Department of Retroviruses and AIDS Research, Pasteur Institute, Paris, France

The identification of genes encoding unique tumor associated antigens (TAAs) has facilitated the development of novel immunotherapeutic strategies in cancer patients. Clinical investigations have focused on targeting these cancer antigens for the generation of anti-tumor T-cell responses. TAA epitopes come from differentiation antigens, from embryonal reexpressed or overexpressed proteins, from mutated proteins and from viral proteins in viraly associated tumors. We have recently developed a novel screening system for identification of immunogenic and antigenic CTL peptide epitopes using $D^{b-/-}X\beta2M^{-/-}$ double knockout mice, transgenic for a single-chain HLA-A2-β2M molecule (HHD mice). Specific CTL were derived by immunization of HHD mice with tumor peptide extracts loaded on antigen presenting cells and with HHD transfected human tumor cell lines CTL induced against peptides from various tumors recognized tumor peptides more effectively than peptides extracted from normal tissues and also reacted with a serie of peptides derived from overexpressed candidate proteins, identified by differential display methods (SAGE, Microarrays) Comparison of CTL derived from HHD mice to CTL induced from patient's PBMC showed overlapping recognition of many candidate peptides. Using these HHD mouse derived CTL we identified novel peptide sequences from Prostate, bladder, breast and colon carcinomas, antigens PAP and STEAP, from Breast Carcinoma antigens MUC1 and BA46-1. Analysis of tumor differentially expressed genes by the SAGE method in colon, followed by screening for HLA-A2 binding peptides resulted in 500 candidate peptides for immunogenicity screening. We have identified 22 antigenic peptides of which 7 peptides were found to be immunogenic in HHD mice. Interestingly 3 of these peptides are derived from the same protein. Differential expression studies, using "DNA chips" were performed on prostate and bladder tumors versus normal tissues. Ten new candidate genes from TCC were analysed for expression and potential immunogenic peptides. Novel peptides from Uroplakins and from MAGE-8 were identified.

Surface plasmon resonance biosensing in the study of viral antigenic sites mimicked by synthetic peptides

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Antigen-antibody binding has been regarded as one of the most representative examples of specific molecular recognition in nature. The simplistic view of antigenic recognition in terms of a lock-and-key mechanism is superseded, since it is now evident that both antigens and antibodies are flexible and can undergo substantial mutual adaptation. This flexibility is the source of complexities such as degeneracy and non-additivity in antigenic recognition. We have used surface plasmon resonance to study the effects of combining multiple amino acid replacements within the sequence of the antigenic GH loop of foot and mouth disease virus. Our aim was two-fold: to explore to what extent can antigenic degeneracy be extended in this particular case, and to search for potential non-additive effects in introducing multiple amino acid replacements. Combined analysis of one such multiply substituted peptide by SPR, solution NMR and X-ray diffraction shows that antigenic degeneracy can be expected as long as residues directly interacting with the paratope are conserved and the peptide bioactive folding is unaltered.

Structural properties of creatine kinase from amphioxus, Branchiostoma belcheri GRAY

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To further our knowledge of creatine kinase (CK) in the fields of molecular evolution and comparative enzymology, we analyzed the CK gene of the protochordate amphioxus. Amphioxus is thought to be the phylogenetic predecessor of vertebrates and thus possesses characteristics, such as enzymological properties, that are associated with ancestral vertebrates.

The results clarified the sequence of 789 bases including the active site. The homology of the active site and the surrounding

48 bases for the amphioxus CK gene to that of the human and electric ray CK-M gene was 89.6% and 87.5%, respectively. The amino acid sequence of this region of amphioxus CK was also identical to that of human and electric ray CK-M. In addition, the estimated secondary structure of amphioxus CK was compared to that of human and electric ray. There were no marked differences in the relative ratio of the α helix, β sheet and turn structures for the peptide structure of CK consisting of 263 amino acid residues. There was a high degree of homology in the sequence of 25 amino acid residues (Met271~His295) near the active site of CK between amphioxus and other organisms, suggesting that this region of CK is functionally essential for transphosphorylation.

The enhanced cell mediated gene transfer into skeletal muscle

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Introduction: Increasing efforts have been focused on skeletal-muscle as a gene transfer target tissue for production of proteins that may be therapeutic for muscle disorders and other system disorders. However, the main hurdle is the inability to efficiently incorporate donor-cell to recipient muscle fibers.

Method: C2C12 was used as donor myoblasts. The marker gene (BMP-4) was introduced into the myoblast, which were then labeled with BrdU. The labeled myoblasts were injected to tibialis anterior of rats together with a peptide mixture containing muscle myogenic factors in test group or with BSA in control. Both groups of rats were sacrificed 5 days after the injections. The tibialis anterior muscles were removed and processed for paraffin sectioning and immunostaining.

Results: Both BrdU labeled nuclei and marker gene expression could be detected in the hybrid tibialis anterior in test group with antibody specific to BrdU and BMP-4. In contrast, there were almost no detection of BrdU labeled nuclei and marker gene expression in BSA control.

Conclusion: This peptide mixture could significantly enhance the incorporation of donor myoblasts into host muscle fibers. Once incorporated, the nuclei and the marker gene would be introduced into the recipient muscle fibers that would express the target proteins in the hybrid muscle fibers.

Molecular dynamics of gelsolin peptides G135–142, G150–169 binding to the PIP2 inserted in lipid bilayer

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[3] Institute of Medicine and Engineering, University of Pennsylvania, Philadelphia, Pennsylvania, USA

Gelsolin is a Ca^{2+}-activated and phosphoinsitide-regulated cytoskeletal actin-binding-and-severing protein, its fragments 135–142: KSGLKYKK (G135–142) and 150–169 KHVVPNEV VVQRLFQVKGRR (G150–169), are responsible for the binding of this protein to actin and the cellular messenger phosphatidylinositol 4,5-bisphosphate (PIP2). The binding of peptides G135–142 and G150–169 to a cluster of four PIP2 molecules in a dimyristoyl-phosphatidylcholine lipid was investigated by means of molecular-dynamics (MD) simulations of 1,600 ps. The binding of the PIP2 molecules to the peptides G135–142, G150–169 showed both electrostatic and hydrophobic nature: lysine residues of the peptides formed salt bridges with the phosphate groups of the PIP2 molecules, while hydrophobic interactions occurred between the nonpolar residues of the peptides and the fatty-acid tails of PIP2. During the binding some of the PIP2 molecules were dragged out of the lipid, thus disrupting the bilayer. After the binding dissociated a draggen-out PIP2 molecule tend to incooporate back to the lipid.

Antiviral activity of 3-hydroxyphthalic anhydride modified proteins

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Division of Applied Physiology, Institute of Veterinary Physiology, University of Zürich, Switzerland

Chemical modification of the proteins: bovine serum albumin, α-lactalbumin, β-lactoglobulin and chicken egg white lysozyme by 3-hydroxyphthalic anhydride (3HP) yielded compounds which exerted antiviral activity *in vitro* as compared with the native unmodified proteins. Of the three enveloped viruses tested: human herpes simplex virus 1 (HSV-1), bovine parainfluenza virus 3 (PI-3) and porcine respiratory corona virus (PRCV), the 3HP proteins were shown to be active against human herpes simplex virus 1 only indicating that a perturbation of the viral envelope is unlikely. Pre-incubation of VERO cells with 3HP-albumin, 3HP-β-lactoglobulin and 3HP-lysozyme resulted in protection against HSV-1 infection whereas pre-incubation with 3HP-α-lactalbumin had no antiviral effect. However, all 3HP modified proteins showed a more significant inhibition when present during or after the viral infection step.

Thus multiple mechanisms appear to be involved in the inhibition of HSV-1 infection. The blocking of cell receptors may contribute to the antiviral activity as shown by the pre-incubation data. However, a direct interaction between the modified proteins and the HSV-1 glycoproteins responsible for viral entry and spread, seems to play a more important role, as indicated by the smaller EC_{50} values obtained during and after the infection.

The regulation of sulphurated amino acids connection. A dummy or a protagonist on the stage of inflammation?

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Amino Acids are usually present in large excess in healthy and the excess is used as source of calories. However, metabolic alterations are observed in ill patients and preferential retention of Sulphur Amino Acids (SAA) occurs during the inflammatory response. The metabolism of Cysteine is modified during the acute phase of sepsis in rats. Sulphate production is lower, whereas the higher liver production of Taurine seems to play a protective role; Glutathione concentration is greater in liver, kidney and other organs and Cysteine incorporation into proteins was higher in spleen, lung and plasma (Acute Phase Proteins) while Albumin level decreases.

Another important phenomenon is the impairment of Methionine conversion to Cysteine during stressed condition. Premature infants or HIV patients synthesise Cysteine from Methionine at a much lower rate. Thus, the metabolic flow through the trans-sulphuration pathway may be insufficient to meet the Glutathione and Cysteine requirement in critical conditions. The pro-inflammatory cytokines, Interleukin-1, Interleukin-6 and TNF-α are the main initiates that alter protein and amino acid metabolism. In this complex picture, SAA supply may contribute to the immune system regulation.

On evolution of HIV – an information theoretic approach

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Sequences obtained from patients infected with human immunodeficiency virus (HIV-1) were analyzed by phylogenetic trees with a genetic measure called entropy evolution rate. In this analysis, we used the third variable (V3) regions of HIV-1 which are classified according to the clinical course of infection. Then we conclude that there exists some relations between the variation of V3 region and the clinical course of AIDS, so that the entropy evolution rate can be one of the measures characterizing the clinical course of AIDS as well as the CD4 counts.

Antioxidant and antihemolytic properties of the cluster rhenium compound with GABA

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Our previous investigations showed some biological activity of newly synthesized cluster rhenium compound – tetrachlorodi-μ-(γ-aminobutirato)dirhenium(III) chloride – I such as antitumour activity, cell-stabilizing activity against osmotic hemolysis, changing of morphology of cells, and other. There exists some information about stabilizing effects of some metal-organic substances with antitumour properties on the isolated ishaemic-reperfused rat heart (Leperre A. 1995) throughout decrease of malonaldehyde (MDA) production. Some new investigations showed the influence of metal-organic substances on apoptotic processes (Winter B. 1998, Syrkin A. 1998), that are considered now as the main mechanism of such tissue damages as ishaemia, myocardial infarct, etc. Thus we tried to analyze such activity of I.

Two models of hemolytic anemia was used: A – on rabbits by introducing of $PbAc_2$ – solutions; this model permits to investigate dynamics of anemia in one experimental animal; B – on rats by introducing of phenylhydrazine chloride. I was administrated as in solution as in lyposomic (lyp) forms. All measurements and models were accomplished according to described procedures.

Administration of I led to: increase of hemoglobin and resistance of erythrocytes and to prolonging of life for hemolytic animals; significant decrease in quantities of MDA and increase in quantities of reduced glutathion (GSH), glutathionreductase (GSR) and glutathionperoxidase (GSP) in myocardium, blood, brain, liver, splenic and entherocites of anemic animals.

The most effective was I in lyposomic form. Mechanism of antioxidant action of rhenium cluster compound is speculated and experiments with some well-known antioxidants to compare with I are working out.

Experimental proof of anti-HIV activity of L-lyzine-α-oxidase

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At present problem of finding remedies against the mostly dangerous human disease – AIDS is one of higher interest.

The aim of this work was the investigation of inhibiting effect of high-pure L-lysine-α-oxidase (LO) E.C.1.4.3.2, extracted from *Trichoderma sp*., on HIV-virus reproduction, comparatively to azidotymidin (AZT), being now in use for treatment of AIDS-patients.

For studying of inhibiting effect of LO, the MT-4 cells, sensitive to citopathical action of virus, were used.

The experimental studying has shown, that the enzyme at concentration 7–70 ng/ml suppresses HIV reproduction and synthesis of virus' proteins, not exerting toxical effect on MT-4 cells. Toxical dose of LO has been determined preliminary. A comparison with standard preparation – azidotymidin, which causes suppression of virus reproduction at concentration 3 mkM (1,2 mg/ml) not exerting toxical effect on MT-4 cells. The same effect is attained having used LO in doses 7–70 ng/ml. Using lower concentrations of enzyme leads to partial increasing of virus' titre comparatively to control cultures.

Obtained data allow to conclude that LO from *Trichoderma sp*. is more high specific agent than azidotymidin, because it needs 1000 times lower concentration for the same action.

Comparison of AZT and LO action on synthesis of virus' antigens presenting in cultural media of MT-4 cells infected with virus, leads to conclusion, that LO has inhibitory action both on virus' reproduction and virus' protein synthesis.

The classification of various organisms according to the free amino acid composition change as the result of biological evolution

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Our previous studies showed that the cellular amino acid composition obtained by amino acid analysis of whole cells, differs in various organisms. These results suggest that the difference in the cellular amino acid composition reflects biological evolution. However, the basic pattern of cellular amino acid composition is relatively constant in all organisms, and the cellular amino acid compositions of the archaeobacteria are quite similar to those determined from codon usage data, based on the complete genomes.

In the present study, the free amino acid compositions in archaeobacteria, eubacteria, protozoa, blue-green alga, green alga, slime mold, plants and mammalian cells were analyzed, to investigate whether changes in their free amino acid compositions reflected biological evolution. Cell homogenates were treated with 80–90% ethanol to separate cellular proteins and free amino acids contained in the cells. Rat hepatoma cells (R-Y121B) were cultured in Eagle's minimum essential medium (MEM) containing 5% serum or in a modified MEM lacking arginine, tyrosine and glutamine. No significant difference in the free amino acid composition was observed between the two cell groups cultured under two different conditions. The patterns of the free amino acid compositions differed completely from those of the cellular amino acid compositions, and from each other in various organisms. Characteristic differences were observed between plant and mammalian cells, and between archaeobacteria and eubacteria. The patterns of the free amino acid composition in blue-green alga, green alga, protozoa and slime mold differed from each other and from those of eubacteria and archaea cells. It has been suggested that the free amino acid composition reflects apparent biological changes as the result of evolution.

Enzymatic production of gamma-glutamyltaurine by gamma-glutamyltranspeptidase from Escherichia coli K-12

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gamma-Glutamyltranspeptidase (GGT) (EC2.3.2.2) catalyzes the hydrolysis of gamma-glutamyl compounds such as glutathione, and the transfer of their gamma-glutamyl moieties to amino acids and peptides. We previously developed enzymatic methods for the synthesis of various gamma-glutamylamino acids using the transfer reaction of GGT from E. coli K-12 as a catalyst. It has been reported that gamma-L-glutamyltaurine has a potent and long-lasting antiepileptic action, and its chemical synthesis has also been reported, but it required protecting and deblocking of reactive groups. Thus, the purpose of this study was to develop an enzymatic method for the synthesis of gamma-L-glutamyltaurine using GGT. The optimum reaction condition was 200 mM L-glutamine, 200 mM taurine and 0.2 unit/ml GGT, pH 10, and 1-hr incubation of 37°C. Forty-three mM gamma-glutamyltaurine was obtained and the yield was 21.%. gamma-Glutamyltaurine was purified by Dowex 1 × 8 column and C18 column, and then identified with gamma-L-glutamyltaurine by NMR and polarimeter.

In this study the yield of gamma-L-glutamyltaurine was comparatively low because synthesized gamma-L-glutamyltaurine was promptly converted into the by-product, gamma-L-glutamyl-gamma-L-glutamyltaurine.

Molecular cloning and recombinant expression of the gene of a antimicrobial peptide from house fly

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The production of antimicrobial peptides is an important aspect of host defense in animals ranging from insects to mammals. They do not target specific molecular receptors on the microbial surface, but rather assume amphipathic structures that allow them to interact directly with microbial membranes, which they can rapidly permeabilize. They are thus perceived to be one promising solution to the growing problem of microbial resistance to conventional antibiotics. Insects express a battery of potent antimicrobial proteins in response to injury and infection. Until now, approximately 170 immune peptides have been characterized from insects and other invertebrates. An antimicrobial gene (Md-cecropin) belonging to cecropin family was cloned from the bacteria-charged adult house fly, *Musca domestica*. expressed in the vector pGEX-4T1. mRNA was isolated and degenerated primers were designed according to the conserved sequences of cecropins. The full-length cDNA encoding Md-cecropin was cloned by RT-PCR and 5′, 3′-RACE and sequenced. The deduced amino acid sequence indicated that a prepeptide with 63 amino acid residues is first translated and then processed to a mature peptide with 40 amino acids. The DNA encoding the mature peptide was subcloned into expression vector pGEX-4T1, and expressed efficiently in E. coli BL21 as a fusion protein. The fusion protein was purified and specifically digested and the Md-cecropin was further purified to homogeneity and the activity spectrum was investigated.

Hyperproduction of phenylalanine by bioengineering Escherichia coli with metabolic engineering methods

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A bioengineering Escherichia coli strain was obtained by metabolic engineering method. Three genes related to the biosynthesis of phenylalanine, aroG, pheA, and tyrB encoded key enzymes: 3-deoxy-D-arabino-heptulonate-7-phosphate synthetase (DS), a bifunctional protein-chorismate mutase (CM)/prephenate dehydratase (PD) and aminotransferase (AT), respectively. In this work, the feedback inhibition of DS and CM/PD were relieved by site-directed mutagenesis on bases of homology comparison of related sequences of the key enzyme. The feedback inhibition resistant genes encoding rate-limiting enzymes in the main and terminal pathways were amplified by co-expressed in order of aroG-pheA-tyrB on the plasmid by their own operator pLpR, PL, and PR. In the recombinant strain showed great resistant to the L-phenylalanine analogues, the specific activities of DS, CM, PD and AT were increased by 3.10, 3.29, 4.91 and 8.16 folds, respectively. As the result, the amount of phenyalalnine biosynthesis of the bioengineered strain was increased greatly compared with that of the host strain.

Proteomics

An enzymatic approach for the mapping of phosphoproteins resolved on two-dimensional polyacrylamide gels

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An enzymatic approach for high-throughput mapping of phosphorylated proteins resolved on two-dimensional (2-D) polyacrylamide gels is presented. Proteins of cultured rat skin fibroblasts were divided into two aliquots, one of which was dephosphorylated using recombinant 1 protein phosphatase. The two aliquots were then subjected to 2-D electrophoresis. The phosphoproteins could be mapped on the 2-D gel by comparing the gels of the phosphatase- and non-treated samples, because the dephosphorylated proteins shifted to more basic positions on the gel. This technique revealed that approximately 5% of the detectable proteins were phosphorylated. Fifteen phosphoproteins were identified by mass spectrometry, including proteasome component C8 and small glutamine-rich tetratricopeptide repeat-containing protein. Furthermore, the extent of phosphorylation of two actin-modulating proteins, destrin and cofilin, was found to be significantly reduced when the cells were chemically or enzymatically detached from the culture dishes. The presented technique can be applied to all biological materials because it requires no protein-labeling step, and is therefore useful for high-throughput mapping of phosphoproteins in proteome research.

Industrial scale high throughput proteomics

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With the completion of the Human Genome sequence MALDI-TOF-MS is increasingly becoming an established method for identification of proteins separated by 2D gel electrophoresis. Mono-isotopic peptide mass fingerprinting (PMF) has been previously shown to be amenable to full automation encompassing the process of acquisition, data processing and databank searching under full software control.

Until now the throughput of MALDI-TOF-MS for proteomics has been limited to several hundred samples in a working day and this represents approximately 5–10% of the total proteins resolved by a large format 2D gel. To reduce the number of proteins to be identified the 2D gels are imaged and analysed to determine differences in expression levels within a set of gels. Although much of the image processing is semi-automated the comparison is labour intensive as manual pattern matching has a role in the gel alignments (land marking). Increased MS sample throughput allows the possibility of identifying every protein spot in a 2D gel within a day. This could eliminate the potentially erroneous step of human gel image alignment, whereby land marking could be achieved using the MS data.

Increased sample throughput requires greater capacity and robust unattended instrument operation. In this poster we describe an integrated robotic multiple plate loader that allows overnight unattended MS operation. Other improvements include an increased laser repetition rate that allows the data capture rate to increase four fold. Sample tracking, data archiving and data reporting are essential attributes of this new technology and these aspects are outlined in the presentation.

The ProteinChip™ Biology System for Ciphergen Biosystems: A novel proteomics platform for rapid biomarker discovery, validation and identification

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The ProteinChip System uses SELDI (Surface Enhanced Laser Desorption/Ionization) ProteinChip technology to perform the separation, mass detection and analysis of proteins at the femtomole level directly from biological samples. Surfaces are based on either chromatographic based chemistry (ion exchange, reverse phase, IMAC etc.) that bind large classes of proteins or biologically defined surfaces (antibodies, DNA, receptors, etc.) that are used to investigate specific protein-interaction events. As with conventional elution chromatography each type of surface is designed to bind a different subset of proteins from a crude mixture. Sample complexity is reduced on the surface by washing with standard biological buffers compatible with the chosen ProteinChip Array. Unlike elution chromatography, proteins are detected directly from the stationary phase using laser based mass spectrometry greatly increasing throughput whilst reducing sample loss and improving reproducibility. Multiple ProteinChip surface and wash conditions are explored with a small sample set to resolve hundreds of proteins and establish assay conditions that reveal candidate biomarkers or diagnostic protein profiles. The resulting custom built assay is then used to monitor disease processes or drug toxicity profiles by screening large banks of samples such as tissue extracts or physiological fluids (serum, urine, CSF, etc.).

Enrichment and proteomic analysis of low-abundance gene products

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To the present, samples representing the total protein mixture have been usually analyzed by proteomics technologies mainly only the abundant, hydrophilic components have been visualized. These proteins could be solubilized with reagents compatible with isoelectric focusing, for example urea and CHAPS. Such an analysis provides us with a limited image of the proteome, which is insufficient for the detection of the majority of the proteins. In a 2-D gel, where about 1 mg of protein amount has been resolved, 1,000–3,000 protein spots can be detected, using Coomassie blue staining. The spots represent the products of only 200–300 different genes. Other gene products, not visualized, are most likely expressed at too low levels for detection or they can not be identified because of limitations of the current technology, they are too small, too large, basic or hydrophobic. Here we will discuss protein enrichment approaches prior to the analysis, which we have applied for the enrichment of bacterial and eukaryotic proteins.

Proteomic analysis of the rat liver mitochondrial proteins

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Subcellular fractionation increases the probability of detection of low-abundance proteins. We prepared mitochondrial, microsomal and cytosolic protein fractions from total liver of male rats. The proteins of the three fractions were analyzed by two-dimensional electrophoresis using broad and narrow pH range immobilized pH gradient strips. The proteins were identified by martix-assisted laser desorption ionization mass spectrometry. In the mitochondrial fraction, 190 different gene products were detected. Approximately 70% of the identified mitochondrial proteins are enzymes with a broad spectrum of catalytic activities. Most of the identified proteins had been detected before in other samples as well, analyzed in our laboratory. Eight gene products were detected for the first time. These were represented by one spot each, whereas most of the frequently detected proteins were represented by multiple spots. In average, approximately 15 spots corresponded to one gene product.

Functional proteomics analysis of signal transduction pathways

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Three kinds of experiments have been carried out successfully in our labs. (1) Identification of post-translational modifications of the endothelin A and B receptors (ETAR and ETBR) including both phosphorylation and acylation. We have developed new, very efficient methods for single step isolation of highly pure ETAR and ETBR from cells. This has allowed us to obtain evidence that the post-translational modifications are very complex and result in multiple phenotypes showing different forms of modification for receptor. As with other systems, e.g. insulin-like growth factors, it is probable that these multiple

phenotypes of the ET receptors correspond to different forms of signalling dependent on cellular state, e.g. the cell cycle. It is, for example, already clear from the phosphorylation of the receptor that a series of different kinases must be involved. (2) Following stimulation of fibroblasts with endothelin, phosphorylation/dephosphorylation signalling cascades involving several hundred proteins have been observed by use of high resolution 2D electrophoresis and detection of phosphorylated proteins labeled with ^{32}P by autoradiography or immunological methods. The large number of proteins involved are being identified by mass spectrometric methods such as mass fingerprinting or sequencing by mass spectropmetry. (3) Differential gene expression has been followed by using ^{35}S Met pulse chase labelling concurrently with endothelin stimulation. At least 50 proteins showed significant changes in expression of 2D gels and these proteins are also being identified. These experiments demonstrate that it is now possible to use proteomics methods to investigate the integration of response to an extracellular signal at the levels of the receptor itself, the subsequent signalling cascades and the ensuing gene expression. The proteomics technology permits concurrent monitoring of large numbers of protein phenotypes (the forms and amounts of individual proteins and is therefore able to provide a global overview of signalling processes which greatly augments more traditional investigations of individual proteins or pathways. Furthermore, these new methods will allow quantitative determination of the changes in protein phenotypes, which is very important in view of the highly non-linear amplification properties of such signalling processes.

An integrated approach to automated high throughput protein identification by 2D gel electrophoresis and mass spectrometry

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Establishing the function of gene products is the major challenge of the post genomic era. The rate-limiting step in this endeavour is the speed with which proteins can be isolated and identified.

Separation of proteins from cell lysates or sub-cellular domains by 2D gel electrophoresis is an established method of visualising these complex systems. Recently mass spectrometry has proved to be a powerful method of further characterising these proteins. From the mass spectrum of the enzyme digest of a 2D gel spot, the resulting digest map is compared with the theoretical maps from the databases and the protein identified when these correlate. MALDI-TOF is of great benefit in these studies since it requires a minimal amount of sample, is relatively tolerant to salts and other contaminants arising from the gel and may be configured for automated sample analysis. High sample throughput with automated analyses including data processing and client-server database searching are already available. Our system automatically acquires the data and processes the MALDI mass spectrum into a monoisotopic peak list. This peak list is then automatically sent to a networked database for protein identification.

When proteins are not identified from the MALDI analysis or an ambiguous result is obtained, then further analysis of the sample by Electrospray CapLC-MS-MS is required. The development of a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (Micromass, Q-Tof) has facilitated the generation of unambiguous amino acid sequences from the MS-MS analyses of tryptic peptides. These MS-MS spectra can be automatically searched against protein, nucleotide or EST databases. Thus enabling protein identification from gel spots, despite non-specific enzymatic cleavage, protein co-migration and post transitional modifications.

For organisms who's genome sequences are poorly represented in the data bases *de novo* amino acid sequencing may be required. Inferring *de novo* peptide sequences from MS-MS data is complex and is often the rate-determining step in this method. However, it is now possible to interpret the MS-MS spectrum automatically. In our approach the raw MS-MS spectrum is reduced to the plausible single-charge, monoisotopic mass spectrum. Sequence interpretation is achieved by generating "trial sequences" consistent with the experimentally determined molecular weight. A probabilistic fragmentation model is used to transform the trial sequences to predicted spectra for comparison to the single-charge, monoisotopic spectrum and to calculate the likelihood that the trial sequence would account for the observed data. The possible number of trial sequences for any peptide is large, for example there are 20^{10} possible sequences for a peptide containing any of the 20 naturally occurring amino acids and having 10 residues. To reduce the scale of the problem a terminated Markov Chain Monte Carlo algorithm is used to produce sequences. This Bayesian method simulates an exhaustive search of all sequences having the correct mass.

The application of a novel precursor ion scanning method for the characterisation of phosphoproteins

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The huge increase in genomic sequence information available, combined with the increased sensitivity and selectivity provided by mass spectrometry, has allowed large-scale protein identification. However the analysis of the post translational modifications present on the identified proteins is a more challenging problem. Currently the approach that offers the most expedient and specific solution, to determine modified peptides, is precursor ion scanning. This approach has primarily been performed on a triple quadrupole mass spectrometer where the rear quadrupole, (MS2) is set to transmit only the fragment ion of interest. The MS1 quadrupole is then scanned across the appropriate mass to charge range.

In this paper we describe a method that allows specific post translationally modified peptides to be identified and sequenced during the course of an HPLC experiment on the Q-Tof mass spectrometer. During the HPLC run the instrument is switched alternately at one-second intervals between low and high collision energy with argon in the collision cell. The quadrupole, MS1 is not mass selective, operating in the rf only mode. The first data set at low energy (4eV) shows only the normal pseudo molecular ions. The second at higher energy shows their fragments. Wherever a product ion of interest occurs in the high-energy data all its possible precursors are revealed by the corresponding 4eV data.

Since the two data sets contain the entire set of precursor and product ions that can be formed it is clearly possible to generate the equivalent of a constant neutral loss scan.

This is invaluable in the case of phosphorylated peptides where the neutral loss of 98da (H_3PO_4) occurs via β-elimination from the phosphoserine and phosphothreonine residues. This allows the Q-Tof mass spectrometer to switch from the MS mode to the MS/MS mode of operation when a potential

pseudo molecular ion exhibits a neutral loss of 98 Da between the high energy and low energy data sets. The product ion MS/MS spectrum can then be acquired on the phosphorylated precursor ion.

In the case of phosphotyrosine, neutral loss of the H_3PO_4 moiety is not observed, however a low mass immonium ion at m/z 216 can be detected. This characteristic ion (from the high energy data) is used to direct the mass spectrometer to fragment potential phosphopeptide precursor ions, which are selected from the low energy data. In this case several precursor ions may require MS/MS interrogation at one decision making time-point.

Automated high throughput protein identification on a hybrid quadrupole orthogonal acceleration time-or-flight mass spectrometer coupled with a MALDI ion source

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With the first draft of the human genome completed large-scale protein identification by mass spectrometry, even for samples originating from higher organisms has become relatively straightforward. This requires a high throughput facility to identify proteins that have usually been separated by 2D PAGE. The approach providing the highest level of automated sample throughput, in terms of samples per hour, is currently MALDI-TOF-MS. This technique provides a peptide mass fingerprint of the protein digests and allows the rapid and accurate identification of the parent protein by comparison to a databank. However, under some circumstances, for example if the number of peptides detected is small or if the sequence coverage is poor, it is advantageous to be able to include even a short piece of sequence information to provide added specificity. In a conventional MALDI-TOF-MS instrument post source decay (PSD) can be used to try and generate sequence information, however this approach is notoriously unreliable in producing good quality MS/MS data. One reason for this is that the peptide ions do not undergo fragmentation in a controlled environment such as a gas cell with selected collision gas and collision energy.

An alternative approach is to use the predictable fragmentation obtained from a hybrid quadrupole ortho MALDI source has been fitted to a hybrid quadrupole orthogonal acceleration time-of-flight (Q-Tof) mass spectrometer. In contrast to a conventional MALDI-TOF-MS instrument the resolution and mass measurement accuracy of the data is comparable between the MS and MS/MS modes. This allows superior data acquisition in the MS-MS mode compared to conventional MALDI-TOF-MS.

A number of modifications have been made to optimise the system for high throughput proteomics. The MALDI source has been configured with a high-density target plate, compatible with a 96 well microtiter plate. The acquisition software has been modified for automated data acquisition in both the MS mode and the MS to MS/MS switching mode. Dedicated processing software has been developed to fully automate the post acquisition and databank searching. this software has been optimised to consider the unique nature of the data acquired from this configuration of instrument.

In this paper we demonstrate the how an MALDI-Q-TOF instrument can be used for high throughput proteomics. We also compare and contrast is functionality in comparison with alternative strategies for high throughput proteomics, namely conventional MALDI-TOF-MS and electrospray LC-MS/MS.

Signal transduction in *Pseudomonas putida* KT2440

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Pseudomonas putida is an ubiquitous, metabolically and physiologically extremely variable soil bacterium. It is kown to be a good colonizer of plant roots and a plant growth promoter. Now, after the sequencing of the total genomic DNA has been finished we have focused on the functional analysis of this strain. Plant growth promotion is achieved in different ways. One is the inhibition of fungal and bacterial phytopathogens, which is known to be a multifactorial mechanism. An important factor of this mechanism is the production of siderophores (iron-transport-agents), small linear or cyclic peptides, which are synthesized in a ribosomal-independent manner by special synthetases. The siderophore production is induced by iron limitation. The regulation of this process was investigated by pulselabelling with [^{33}P] inorganic phosphate. 2D-protein patterns generated from cells grown with and without Fe-supplementation were compared. Proteins which were phosphorylated under iron limitating conditions were analysed by MALDI-TOF peptide mass fingerprint. For the identification of the proteins we used an in-house peptide mass database which has been built based on the genomic sequence data.

Contextual integration of proteome bioinformatics in the WorksBase software system

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WorksBase Software for Proteomics is a platform independent information management system encompassing laboratory experimental workflow and bioinformatics for protein and biochemical research. The WorksBase system is designed to allow direct internal integration between laboratory experimental data and background biological knowledge found in reference and in-house data, such as gene, protein and functional annotation databases. WorksBase provides a cross-disciplinary research infrastructure for drawing together multiple lines of evidence for characterization of proteins, and integration of this data with domains such as gene expression, pharmacological screening, structure and related areas. While the focus is on the biology underpinning the experimental work, the system is also designed with the capability of providing a sample and workflow tracking system for use in the wet lab, effectively a proteome LIMS (laboratory information management system).

As experimentation proceeds in the laboratory, WorksBase software can be used for development of hypotheses on protein, biochemical pathway, and post-translational processing involvement in biological systems and disease processes. As such, identifications that are derived from lab work and user observation can be used to augment the reference data repository. However, unlike databases ands systems where the methods and reasoning for assignment of annotations are obscure, by maintaining the link between the source data and the biological roles derived from them, the accuracy and integrity of any information stored in the WorksBase system can be directly ascertained.

Changes in the brain protein levels following administration of kainic acid

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Kainic acid (KA), a potent neurotoxin and excitatory amino acid, leads to derangements and modulation of brain proteins. No global brain protein expression pattern induced by KA-treatment has been reported yet. We studied the effect of systemic KA administration on the levels of brain proteins. Rats were injected placebo or KA intraperitoneally and brain was taken after one week. The mitochondrial and cytosolic fractions of the brain proteins were analyzed by proteomics technologies. Heat shock protein HSP 27 was exclusively detected in brains of animals treated with KA. The levels of neurofilaments and alpha-internexin were significantly decreased and a fragment of tubulin alpha-1 chain was manifold increased in KA-brains. The mitochondrial enzymes dihydrolipoamide dehydrogenase, ATP synthase beta chain and isocitrate dehydrogenase were reduced and pyruvate kinase M1 was increased following KA treatment. The results indicate altered regulation of heat shock proteins, neuronal death, cytoskeletal disruption and mitochondrial derangement by systemic KA administration. This report confirms and extends previous studies on the effect of KA on the expression of brain proteins and suggests that our analytical system can serve as a model for neurotoxicological, neurobiological and neuropathological proteome studies.

The rat brain mitochondrial proteins

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We constructed a two-dimensional database for rat brain mitochondrial proteins. Rat is a useful model of human diseases of the central nervous system. In order to detect alterations in the levels of the low abundance brain proteins, the mitochondrial, microsomal and cytosolic fractions were prepared. The proteins of each fraction were analyzed by two-dimensional electrophoresis, followed by martix-assisted laser desorption ionization mass spectrometry. Approximately 500 proteins were identified in the mitochondrial fraction, which were the products of 165 different genes. About 75% of the identified proteins were detected in the mitochondrial fraction only and the rest were detected in the cytosolic and about 2% were found in the microsomal fraction as well. 98 of the 165 proteins had not been detected before in our laboratory. The identified proteins were in the majority enzymes or enzyme subunits with a broad spectrum of catalytic activities and heat shock proteins.

Automated software for identification and relative quantification of differentially expressed proteins from isotope coded affinity tag (ICAT) LC-MS data

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Whilst LC-MS/MS has been utilised for the identification of proteins from complexes and cell lysates (qualitative proteomics), the quantitative study of gene expression using differential display has until recently been the preserve of a 2D gel based proteomic experiment. However, recently a great deal of interest has been generated on the use of isotope coded affinity tags (ICAT) for the quantitative study of gene expression at the proteome level. The technique is based upon chemically modifying the cysteine residues of proteins isolated from cells in two different states with light and heavy isotopically labeled reagents. The two cell states are then combined, digested with trypsin and the cysteine containing peptides preferentially selected by binding to an avidin column, prior to analysis by mass spectrometry. The eluent from this column is then analysed by capillary LC ESI-MS/MS. Interrogation of the eluting peptides by tandem mass spectrometry and databank searching results in the identification of the associated protein.

We describe how ICAT data analysis has been automated within a software environment. The MS and MSMS data acquired using the QTof instrument are processed and analysed using a new algorithm which recognises related isotope clusters and quantifies their relative intensities. Based on a user defined ratio threshold the software will automatically carry out an LC-MS/MS experiment and databank search in a client-server mode and provide a report of the identified proteins and their expression ratio in the two cell states.

Deterioration of the transcriptional, splicing and elongation machinery in brain of fetal Down Syndrome

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Perturbation of brain development i.e. regulation of gene expression, differentiation, growth and migration in Down Syndrom (DS) has been reported to occur early in life pointing to impairment of the complex system of transcription and or translation and indeed, altered expression of transcription factors has been reported in adult DS brain. We therefore decided to compare the transcriptional and translational machinery in cortex of brains of controls and fetuses with Down syndrome in the second trimenon of gestation. We determined a series of transcription/translation factors by 2 D-electrophoresis followed by MALDI – identification and quantification with specific software.

The protooncogene C-CRK, CRK-like protein, elongation factor 1-alpha 1, elongation factor 2, elongation factor tu and two out of four spots representing PTB-associated splicing factor PSF were significantly downregulated in brain of fetal DS fetuses as compared to controls.

The finding of reduced transcription and translation factors may indicate deranged protein synthesis. The underlying cause for individual reduced transcription, splicing and translation factors may be explained by chromosomal imbalance or by posttranslational modifications as e.g. phosphorylation, known to be aberrant in DS. Reduced expression of transcription factors in fetal DS during early life may be responsible or reflecting impaired brain development and deficient wiring of the brain in DS.

Proteome analysis of *Acinetobacter radioresistens* reveals proteins involved in aromatic compounds uptake and degradation

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A fast phenol degrading *Acinetobacter radioresistens* strain was isolated in our laboratories and selected for bioremediation applications. This bacterium is also able to grow on benzoate and catechol as sole carbon-energy sources, metabolizing them *via* the *ortho* route. In previous researches we detected, by means of proteome analysis, some marker enzymes of the phenol and benzoate degradative pathways. In the present work we extend the identification of the proteins involved in the aromatic-ring opening (the different components of the phenol hydroxylase and benzoate dioxygenase, the catechol dioxygenase isozymes) together with other satellite proteins specifically induced by the aromatic growth substrate. Of these last proteins some are probably related to the cellular uptake of benzoate and phenol while others are ascribed to the GroEL family of heat-shock chaperonines, involved in proteins processing and folding. Aromatic substrates may thus act as stress-agents like heat or cold.

Proteomic studies on rat body fluids

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Previously, we have characterized rat serum proteins, both under "normal conditions" and during experimental inflammation, using two-dimensional electrophoretic separation, densitometric quantitation and identification by mass spectrometry and immunological procedures (http://linux.farma.unimi.it/homeframed.html). We have now extended these studies to the protein composition of cerebrospinal fluid (CSF) and urine, and have identified several proteins specific to these fluids, including major urinary protein, uromodulin, and prostaglandin D synthase. These baseline data provide a useful comparison to the biological fluids of stroke-prone spontaneously hypertensive rats, an inbred strain, which develops cerebrovascular abnormalities following high blood pressure. Our studies have detected signs of an inflammatory condition several weeks prior to stroke. We have confirmed the sharp rise in proteinuria preceding stoke onset, and have identified the excreted proteins. Following stroke we observe a massive increase in CSF protein concentration as serum proteins, even those of large molecular size, cross an impaired blood-brain barrier.

Quantification of low molecular weight urinary proteins using high resolution two-dimensional electrophoresis and mass spectrometry

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As a first step to discover useful disease markers from the urinary proteome, we have developed a unique and systematic approach for detection of low molecular weight urinary proteins by using high resolution two-dimensional (2D) electrophoresis and mass spectrometric methods. Unlike previous studies on urinary proteins, and most importantly as observed in present study, our results show that a large number of low molecular weight protein spots can be visualized in the 2D electrophoresis pattern. It was observed that protein concentration and fractionation methods were critical for our ability to detect many proteins in the gel pattern. Therefore, several approaches were carefully considered to concentrate and fractionate proteins in urine samples. Initially, urine specimens from normal individuals were concentrated by using centrifugation and ultrafiltration methods. The concentrated samples of urine proteins were then fractionated by size exclusion and immunoaffinity chromatography. The size exclusion method was used to generate two fractions of proteins based on their native molecular weights. Further, this method allowed us to enrich concentrations of less abundant proteins for each fraction. The immunoaffinity method was used to specifically remove well-known abundant urinary proteins (such as albumin) from the above mentioned two fractions. That the 2D pattern includes many native low molecular weight proteins was confirmed by analyzing both protein fractions from size exclusion chromatography. A detailed mass spectrometric analysis of the protein spots is carried out to identify the proteins observed in 2D pattern. Since urine is an ultrafiltrate of plasma, many factors in urine are present in proportion to their rate of synthesis in the body. These factors include many low molecular weight proteins that remain undiscovered due to their low abundance. Therefore, the present analysis of urinary proteins would serve as the most useful guide for the discovery of novel diagnostic markers in urinary proteins.

Proteomics of breast cancer cells and tissue: glycolytic enzymes and chaperonins, old proteins, new markers?

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Breast cancer is one of the leading causes of death for cancer among women. There are different types of breast cancers, grouped as invasive and non-invasive types. Among the invasive types "infiltrating ductal carcinoma" (IDC) accounts for about 80% of all breast cancers. In order to study some biological properties related to this type of cancer, we have developed and well characterized an "in vitro" system, consisting of an IDC-derived cell line, 8701-BC (Minafra et al., Br. J. Cancer, 60, 185-192, 1989) and some of its cloned cell lines, selected for their high and low invasive activity in matrigel. Using this model we are producing proteomic maps to compare with that of non-tumoral breast epithelial cells and with breast tissue fragments, existing in our collection or available at the ExPASy proteomics server. Protein identification is currently done by means of gel matching, Edman-microsequencing and immuno-detection. To rationalize data we grouped proteins into functional categories: a) cytoskeletal proteins, b) metabolic enzymes, c) chaperonins and other functionally related proteins, d) peptides and enzymes with regulatory functions. A fifth group consists of peptides with unknown identity. Among these sets of proteins we found that glycolitic enzymes and some chaperonins are overexpressed in cancer cells. In addition, new isoforms of potential interest as

biomarkers for breast cancer, were identified by means of microsequencing.

Proteomics of human cytomegalovirus

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Human Cytomegalovirus (HCMV) is an ubiquitous virus, belonging to the Herpesviridae family, Betaherpesvirinae subfamily, able to induce morbidity in immunocompromised patients and congenitally infected new-borns. HCMV has the largest genome among the herpes-viruses (240 kbp): AD169 strain genome was completely sequenced, containing about 200 open reading frames encoding polypeptides, most of which are not characterized. The viral genes are activated in a cascade fashion: 1) alpha, immediate-early genes, coding for regulatory proteins necessary for the activation of 2) beta, early genes, needed for DNA replication, and, finally 3) gamma, late genes, coding for structural proteins of the mature virions. This latter category includes the virus surface antigenic proteins responsible for the main immune response during HCMV infections. Although the sequencing of HCMV genome has been completed, very little is known about the actual nature of the viral proteins. The most appropriate approach to characterize HCMV phenotype is to study its protein expression as it is carried out within the host cell. For this purpose, we analyzed by two-dimensional electrophoresis (2D-PAGE) the protein phenotipic repertoire of human fibroblasts and compared it with that of the same cell type following infection with HCMV strain AD169. The phenotypic 2D map of human fibroblasts dramatically changes following infection with HCMV. A relevant amount of newly appeared spots is attributable to HCMV proteins, mainly of the structural category, since we analyzed host cells at the 7–9th day of infection, when the late, gamma genes are supposed to be the only to be activated. On the other hand, a marked decrease of protein synthesis can be easily evidentiated in the infected fibroblasts respecting to uninfected cells. A temptative mapping of the main structural viral proteins (those against which patients sera are directed) was carried out by immunoblotting, microsequencing and mass spectrometry.

Comparative proteomics of cultured cells: Identification of genetic defects and molecular mechanism of apoptosis regulation

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We employed a comparative proteomics of cultured cells to study mechanism of genetic disorders and for identification of key proteins involved in cell proliferation, differentiation, and death. In particular, this technology proved to be very useful to understand molecular basis of severe inherited diseases resulting from deficiency of lysosomal membrane transporters, and a role of programmed cell death (PCD) of vascular smooth muscle cells (VSMC) in cardiovascular disorders. To increase sensitivity of the identification of cellular proteins we have either have isolated cellular organelles such as lysosomal membranes or performed the differential extraction of soluble, membrane and cytoskeletal proteins. By comparison of proteomic cell maps from normal controls and individuals affected with lysosomal transport disorders we have selected and identified several candidate disease-causing proteins, which have to be further studied by mutation analysis and functional expression. For the second group of disorders we identified proteins, which de-novo synthesis could result in survival of VSMC including a two members of hsp70 family, a molecular chaperone GRP78, and so-called mortalin (GRP75) highly expressed in non proliferative tissues and associated with mortal cell phenotype.

The analysis of complex tryptic peptide mixtures by multi-dimensional LC-MS/MS on a hybrid quadrupole orthogonal acceleration time-of flight (Q-Tof) mass spectrometer

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Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is the established technology employed for the separation of proteins from a cell lysate, sub-cellular organelle or tissue sample prior to identification of the excised protein spots by mass spectrometry. In the order of several hundred to several thousand proteins, can be separated and visualised on a 2D gel by conventional staining or utilising fluorescent labelling techniques. The advantage of performing a two dimensional gel based separation is the ability to obtain quantitative information by comparing and contrasting two samples in a differential display experiment, for example, between a healthy and diseased state. The last stage however stipulates that the gels are reproducible which can be both difficult and time consuming to achieve. The relativity poor dynamic range that the gels exhibit also limits quantification. Other restrictions include the under representation of certain classes of proteins, such as membrane proteins, large or small proteins and very acidic/basic proteins. For these reasons, amongst others, alternatives to 2D-PAGE are being investigated.

Advances in both LC and mass spectrometry instrumentation have allowed the analysis of protein complexes, which have not been separated on a 2D gel. In this case protein identification is achieved via database searching of ESI-MS/MS data. This provides qualitative information on the proteins that are present and has recently been coupled with isotope dilution experiments to provide relative quantiative information. These experiments normally involve separation of the complex digest mixture by microcapillary liquid chromatography connected to an instrument capable of data dependant switching between the MS and MS/MS modes. Using this approach it has been demonstrated that hundreds of MS/MS spectra can be acquired in a fully automated fashion, resulting in the identification of significant numbers of proteins, including low copy number proteins, from a single LC-MS/MS experiment.

If, however, a complex protein mixture is to be investigated then a fractionation step prior to separation of the peptides on the basis of their hydrophobicity would be advantageous. We have, therefore, adopted a 2D LC-MS/MS approach using a capillary LC system (CapLC) operating at nanoliter per min flow rates coupled to a Q-Tof 2 mass spectrometer. By replacing the standard sample loop within this system with a strong cation exchange (SCX) cartridge followed by a C18 trap cartridge it is possible to pre-fractionate the peptides before separation on a C18 column. After loading the sample, discreet fractions are sequentially eluted from the cation exchange cartridge using a salt step gradient; the eluted peptides are then retained on the trapping C18 cartridge whilst they are desalted. Finally the peptides are eluted from the C18 pre-column,

at 200 nL/min, onto a 75 uM ID × 10 cm Waters Symmetry analytical column for separation and elution into the mass spectrometer.

This analytical approach will be discussed with examples where this methodology has been used for the analysis of standard protein mixtures and also for the analysis of cell lysates and sub-cellular fractions.

Electrophoretic analyses of monoclonal IgG of different subclasses

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Monoclonal IgG are commonly observed in various B cell disorders, the most clinically relevant being multiple myeloma. In a series of 73 serum samples, immunofixation identified IgG_1, IgG_2, IgG_3, and IgG_4 in 63, 4, 5, and 1 cases, respectively. Their light-chains were κ in 45 cases and λ in 28 cases. These monoclonal IgG were further characterized by high resolution two-dimensional polyacrylamide gel electrophoresis (2-DE) with various isoelectric focusing conditions as well as by 3-DE (2-DE of the proteins extracted from agarose after serum protein agarose electrophoresis). After 2-DE or 3-DE, the monoclonal γ-chains were not visualized in 29 out 73 cases, whatever the isoelectric focusing conditions that were tested. In 6 cases, γ-chains were only detected using alkaline pH 6–11 gradients. Monoclonal γ-chains and light chains were highly heterogeneous in terms of p*I* and M*r*. However, a good correlation ($P < 0.05$) was observed between the index of migration of the monoclonal IgG in agarose gels and the p*I* of their γ- and of their light-chains ($R = 0.735$, multiple linear regression). Because of the extreme diversity of the different γ-chains as well as of the κ- and γ-chains, it appears that a classification of monoclonal IgG based only on their electrophoretic properties is not possible.

Deranged expression of molecular chaperones in brains of patients with Alzheimer's disease

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Alzheimer's disease (AD) is one of disorders caused by protein conformational changes and recent studies have shown that several chaperone proteins are involved in this process. As information of chaperone expression in AD brain is limited, we aimed to study the expressional pattern of chaperones in several brain regions as this may be essential to understand how folding defects can lead to disease. We studied the concomitant expressional patterns of molecular chaperones in seven brain regions of adults with AD using two-dimensional polyacrylamide gel electrophoresis (2-DE) and matrix-associated laser desorption ionization mass spectroscopy (MALDI-MS). We unambiguously identified and quantified nine different chaperone proteins. Six chaperone proteins, heat shock protein 60 (HSP 60), HSP 70 RY, heat shock cognate (HSC) 71, alpha crystallin B chain, glucose regulated protein (GRP) 75 and GRP 94 showed aberrant expressional patterns depending on brain region. HSP 70.1, GRP 78 and T-complex 1 (TCP-1) epsilon subunit did not show any significant expressional change. These findings are compatible with neuropathological and biochemical abnormalities in AD brain and this report presents the first approach to quantify nine different chaperones simultaneously at the protein level in individual AD brain regions providing evidence for the relevance of aberrant chaperone expression to AD neuropathology.

Data directed real time instrument feedback and control: Creation of dynamic peptide include and exclude lists from on-the-fly databank searching

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The mainstream approach to protein separation, visualisation and identification has been to use two-dimensional gel electrophoresis coupled to mass spectrometry for the identification of the separated proteins. However this approach is limited with the level of protein that may be loaded onto the 2D gel and the nature of the proteins that may be incorporated onto the first dimension (IpG strip).

An alternative approach for the qualitative analysis of complex protein mixtures is the use of tryptic digestion followed by electrospray LC-MS/MS. This approach is dependent on a high degree of chromatographic separation prior to the mass spectrometer, such that ideally individual peptides are eluted into the source. If this is the case then the dynamic range of protein identification can be increased and low copy number proteins can be identified. Often, however there is a large degree of redundant sequence information acquired, as in theory one peptide MS/MS spectrum is sufficient to identify a protein from a sequence databank. If a protein identification is obtained from a databank search of an MS/MS spectrum, it is potentially valuable to exclude the rest of the theoretical tryptic peptides to "mine" deeper into the protein complex being studied.

We have introduced a new protein databank search engine capable of matching a tryptic peptide from the Swissprot/TrEMBL databank to an MS/MS spectrum in one second. Using this search engine we are able to generate dynamic tryptic peptide exclude and include lists, based upon the theoretical tryptic peptides from the identified protein, which can be passed to the acquisition software of our Q-Tof mass spectrometer in real time. Thus, we are able to automatically steer the Q-Tof, during acquisition, to select and switch to the MS/MS mode only on those peaks that meet the modified selection criteria. Experiments can be designed in which peaks that belong to a protein already identified during acquisition can be avoided. This exclusion list is based upon m/z, charge state and a user definable mass tolerance. The mass measurement of the data from the Q-Tof mass spectrometer is typically better than 10 ppm and as a consequence of this a tight mass tolerance can be selected, thus making the exclude list extremely specific. Alternatively, in the case of samples derived from 2D gel spots, the mass spectrometer may abandon the current sample, re-equilibrate the LC column and move on to the next sample.

To illustrate this methodology we show examples, both on standard samples and complex protein mixtures where Q-Tof data acquisition has been directed based upon the results from a databank search. This data will be compared and contrasted to data acquired in the normal automated LC-MS/MS mode.

Medicine

The specific anti-cancer activity of green tea (−)-epigallocatechin-3-gallate (EGCG)

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The effect of the green tea polyphenol-(−)epigallocatechin-3-gallate (EGCG) was tested in cultures of normal and transformed NIH-pATM*ras* fibroblasts. In this system transformation can be induced at will by the addition of dexamethasone, which induces the expression of H-*ras* by activating the mammary tumor virus long terminal repeat (MMTV-LTR) promoter. This facilitates a reliable comparison of the susceptibility of normal and transformed cells to EGCG. It has been shown that EGCG inhibited the growth of transformed but not of the normal fibroblasts. In an attempt to elucidate the mode of the preferential inhibitory activity of EGCG, its effect on growth promoting factors has been examined. The level of ornithine decarboxylase (ODC, EC 4.1.1.17), which is a signal for cellular proliferation, was reduced by EGCG in the transformed but not in the normal cells. EGCG also showed strong inhibition of tyrosine kinase and mitogen-activated protein kinase (MAPK) activities, without affecting the kinases in the normal cells. Similarly, EGCG also preferentially decreased the levels of the oncogenes Ras and Jun in transformed cell. EGCG preferentially induced apoptosis in the transformed fibroblasts. *In vitro* chemosensitivity tests demonstrated that EGCG inhibited the proliferation of leukemic cells. These findings suggest that EGCG has a therapeutic potential in the combat against cancer.

L-Methionine: Immune supportive supplement in HIV+ patients: A South African study

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Objectives: To develop a safo, affordable immune supportive therapy for HIV+ patients.

Design: A randomised, double blind, placebo-controlled study, testing an internationally patented L-Methionine combination (LMC), in approximately 400 HIV+ patients; not yet on anti-viral treatment (CD4 count 200 to 500).

Methods: Parameters measured included: CD4 count, total lymphocyte court, viral load, several clinical, as well as mechanistic parameters. The difference in the change from the baseline (Active – Placebo) was determined for each parameter. The study is ongoing.

Results: Within 3 months, significant trends are noted. The CD4 count of the patients on the active therapy, presented with a slower rate of decrease, compared to the placebo group, mean difference (MD) in this change from baseline; 15.1/cmm and 95% confidence interval (C1), this was confirmed by the total lymphocyte court values. After 12 months the placebo group was placed on active, causing the difference to disappear.

Conclusions: Although further trials are needed, these results already indicate T-Methionine as an important role player in the immune system of patients with impaired immune function.

Covariation of plasma sodium and taurine in critical illness

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In critical illness and sepsis, changes in amino acid plasma levels (AA*pl*) have been assessed extensively, while little is known about the relationship with changes in other plasma components, such as those involved in fluid-electrolyte and osmotic balance; their investigation is also limited, in large clinical samples, by inter-patient variability. We analyzed the relationships between plasma sodium (Na^+pl, mEq/L) and AA*pl* (μM/L) in eighty consecutive measurements performed in one single patient with post-traumatic sepsis and severe, prolonged illness. Unique feature of plasma taurine (TAU) was maintenance of a highly significant inverse correlation with Na^+pl ($r^2 = 0.48$, $p < 0.001$). All other AA*pl* were correlated directly, or unrelated, to Na^+pl, the only exception being a weak inverse correlation between tryptophan and Na^+pl. TAU was correlated, strongly and directly, also to phosphoethanolamine (PEA), glutamate (GLU) and aspartate (ASP):

$TAU = 707.1 + 7.3(PEA) - 4.6(Na^+pl)$
$r^2 = 0.86$, $p < 0.001$
$TAU = 710.2 + 11.4(ASP) - 4.7(Na^+pl)$
$r^2 = 0.61$, $p < 0.001$
$TAU = 677.4 + 30.7(\log GLU) - 4.7(Na^+pl)$
$r^2 = 0.59$, $p < 0.001$

and unrelated, or weakly and inversely related, to other AA*pl* (measurements of beta-alanine were not included).

Co-variation of Na^+pl and these AA*pl* (particularly TAU and PEA) was influenced by severity of illness, and more complex regressions were needed to quantify this effect.

These results provide useful information on interdependency of TAU, Na^+pl and other AA*pl* in critical illness.

Relationship between Parkinson's disease and plasma alpha-tocopherol concentrations

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The central nervous system (CNS) shows an exceptionally high degree of vulnerability to reactive oxygen species. Considerable evidence suggests that free radical formation and oxidative stress might play an important role in the pathogenesis of Parkinson's disease (PD). Moreover, it has been reported that the levels of glutathione and vitamin E increase in the brain of patients with PD as a compensatory mechanism to deal with oxidative stress. Since vitamin E is an effective free radical scavenger in the brain, its neuroprotective function is the issue of new therapeutic approaches in neurodegenerative diseases. To elucidate the possible role of vitamin E in the pathogenesis of PD, we assessed the plasma levels of vitamin E, measured by high-performance liquid chromatography, in 54 patients with PD. Vitamin E concentrations were also assessed in 93 age and

sex matched normal individuals. The mean plasma levels of vitamin E did not differ significantly between these two groups (22.5 ± 8.15 mmoli/l for PD patients and 21.0 ± 7.9 mmoli/l for controls). The results of our study suggest that plasma vitamin E concentrations do not play a major role in the pathogenesis of PD.

Vitamin E and cardiovascular disease: nutritional and intervention approaches

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Vitamin E is represented by a family of eight natural vitamers (4 tocopherols and 4 tocotrienols) of which α-tocopherol (α-T) form has the highest biological activity. This vitamin accounts for most of the lipid-soluble, chain-breaking antioxidant activity in mammalian tissues and plasma. In addition, it shows nonantioxidant properties through which it modulates cell signaling and the expression of specific enzyme in cell models playing a role in atherogenesis (e.g. endothelial and inflammatory cells).

The preventive effect of vitamin E on ACDV is still a matter of debate. The largest epidemiological investigations and 4 out of 5 main intervention studies at yet available have suggested a correlation between levels of vitamin E and incidence of atherosclerotic cardiovascular disease (ACVD) and related mortality. An overall conclusion rising from these studies is that the major effect (if any) of vitamin E is to be found with intakes higher than 100 IU (100 mg all-rac α-tocopheryl acetate) per day. However, other investigations have failed to demonstrate a beneficial effect of vitamin E against ACVD, suggesting the need for more studies on its metabolism and function.

Recently a family of tocopherol binding and transport proteins has been identified. They play a key role in the selective uptake and delivery of tocopherols to lipoproteins and tissues. Genetic abnormalities of these proteins have been demonstrated to be responsible for conditions of vitamin E deficiency in humans. Their tissue distribution and regulation are now under investigation.

The information available on vitamin E metabolism and its response to supplements or diet changes are at yet poorly characterized. The synthesis of stable isotopes and the characterization of major metabolites of main vitamers provide important advances in this research. In the last years, both plasma levels and urinary excretion of relevant metabolites of α-T have been characterized. Little information is available on metabolites formed by other vitamers. The emerging role of γ-T and its main catabolite 2,7,8-trimethyl-2-(b-carboxyethyl)-6-hydroxychroman (γ-CEHC) in the defense against nitrogen oxide species formed during the activation of inflammatory cells is now well established and suggests the need for further studies on the bioavailability and transformation of this homologue of vitamin E in humans.

At the same time, an oxidation byproduct of α-T found in human plasma, namely α-tocopherylquinone, has been proposed to be also *de novo* synthesized from phenylalanine with a role in the genesis of a defective polyunsaturated fatty acid metabolism observed in phenylketonuric patients. This suggests a possible, and at yet unexplored relationship between vitamin E and phenylalanine/fatty acid metabolism which might have also a role in atherosclerotic process.

Methionine and antioxidant status in patients undergoing mars treatment

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Four patients (3 females, 1 male; aged from 21 to 45 years) affected by severe liver failure, were treated by a new blood purification method, namely Molecular Adsorbent Recycling System (MARS). MARS removes albumin-bound toxins using a specific membrane with a dialysate solution containing albumin. In the patients the plasma levels of Methionine (Meth), branched chain and aromatic amino-acids and liposoluble antioxidants were measured. The Fischer's Index did not show any significant variation, whereas the plasma levels of Meth were well correlated with the levels of liposoluble antioxidants (Vitamin E and CoQ10). In fact, in the patients receiving just branched chain amino-acids, the plasma levels of both Meth and antioxidants progressively decreased. On the contrary, if Meth and branched chain amino-acids were administered, the plasma levels of CoQ10 and Vitamin E showed a positive correlation with the plasma Meth levels ($p < 0.02$; $r = 0.63$ and $p < 0.005$; $r = 0.77$, respectively). Since Vitamin E and CoQ10 are mutually dependent-molecules, the administration of Meth, essential substance for CoQ10 synthesis, may be effective to maintain a good antioxidant status in patients with severe liver failure undergoing MARS treatment.

Obtaining and ophthalmologic application of novel synthetic peptide epitalon regulating the retinal functions

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We obtained new synthetic peptide preparation Epitalon to be widely applied as a pharmaceutical due to its properties important in medical care. Epitalon was found to stimulate repair processes in retinal diseases via restoring the retinal functions, in particular its photoreceptors. This promising peptide drug is a linear tetrapeptide of formula H-Ala-Glu-Asp-Gly-OH (alanyl-glutamyl-aspartyl-glycine). The substance was obtained by classic peptide synthesis in a solution (scheme: (1 + 2) + 1) with N-oxysuccinimide activated esters. COOH-groups of lateral radicals of glutamic and aspartic acids were defended as benzyl esters, benzyloxycarbonyl (Ala) and tert.butyloxycarbonyl (Glu) N-defending groups were employed, deblockade conducted by trifluoroacetic acid and catalytic hydrogenolysis. Preparative HPLC on a reverse phase was applied for purification. The product was fully characterised by the data of analytical HPLC (substance content – 99%), amino acid analysis, IR- and HMR-spectra. The ready drug form is ampoules containing 10 μg of the substance in 1 ml of isotonic solution. Epitalon application in patients with pigmented retinal degeneration stopped the pathology development in 100% and increased visual functions in 80% of the cases. In 70% of the patients visual acuity raised by 0.1–0.3. Electroretinography confirmed the retinal functional activity increase.

Expression of apoptosis related proteins in brains of patients with Alzheimer's disease

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An increasing number of proteins are implicated in apoptosis and several of them have been shown to be altered in Alzheimer's disease (AD) brain. Because of this apoptosis is thought to be the underlying mechanism of neuronal cell loss in AD. To further substantiate this hypothesis we investigated the expression of a recently identified apoptosis related proteins and other apoptosis regulators in frontal cortex and cerebellum of AD by western blot and ELISA techniques. Quantitative analysis revealed unaltered levels of Bax and RAIDD (Receptor interacting protein associated ICH-1 (caspase-2)/CED-3 (*Caenorhabditis elegans* death protease-3)-homologous protein with death domain) in both regions. ZIP (Zipper interacting protein) kinase, Bim/BOD (Bcl-2 interacting mediator of cell death/Bcl-2 related ovarian death gene) and p21 were significantly increased only in AD frontal cortex ($P < 0.05$, in all cases). Cerebellar Bcl-2 levels were significantly increased in AD ($P < 0.01$) while in AD frontal cortex, although the levels tended to increase did not reach significance level. The results indicate that apoptosis indeed account for the neuronal loss in AD. However, it does not seem to involve Bax and RAIDD.

Studying the role of the citrullin-containing epitopes of filaggrin in rheumatoid arthritis

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Rheumatoid arthritis (RA) is a systemic autoimmune disease of unknown etiology. It is the most common of the inflammatory joint diseases, affecting 1–2% of the world population.

Anti-filaggrin antibodies (AFA) directed against the epidermal protein, filaggrin, belongs to the most specific markers of RA. Epitopes, containing citrulline within the sequence of filaggrin, have been recently identified as major antigenic sites recognised by AFA.

The aim of our study was to identify these epitopes of filaggrin derived-peptides targeted by RA specific antibodies to provide further information about the nature of the initial autoantigenic substance.

The most immunogenic six sequences of filaggrin and further, on the N- and C-terminal, shortened version of the original peptide (^{306}SHQESTRGRSRGRSGRSGS^{324}) were synthesized. We used conventional solid-phase peptide synthesis (Fmoc strategy) carried out on "MULTIPIN NCP" non-cleavable kit. In ELISA experiments the presence of AFA was deter-mined using serum samples of RA patients and healthy blood donors.

In conclusion our results provide further evidence that not simply the presence of citrulline but also the nature of its surrounding amino acids have important role in the creation of autoantigenic epitope reactive with anti-filaggrin antibodies.

Lack of association between IL-1alpha, IL-1beta and IL1-receptor antagonist polymorphisms and Multiple Sclerosis

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The autoimmune nature of Multiple Sclerosis (MS) has introduced cytokine genes as logical candidates for the loci determining susceptibility to the disease and/or influencing disease progression. Interleukin (IL)-1alpha and 1beta are major proinflammatory cytokines that have been related with several chronic inflammatory diseases such as MS. The IL 1-receptor antagonist (IL-1RA) is a protein structurally related to IL-1beta that effectively inhibits the proinflammatory effects of IL-1. A polymorphism in the 5′-flanking regulatory region at −889 of the IL-1alpha gene, which may cause an overexpression of IL-1alpha and a variable number tandem repeats (VNTR) polymorphism in the IL-1RA gene have been also associated with several inflammatory diseases. Two biallelic base change polymorphisms in the IL-1beta gene have been reported to influence the protein production: one is located in the promoter region at position −511 and the other is in exon 5 at position +3953. To analyze the contribution of IL-1alpha, IL-1beta and IL-1RA genes in the genetics predisposition to MS, we have examined four polymorphic genetic markers in 132 Italian patients with clinically definite MS and 130 healthy controls. In summary, no significant differences in genotypes and allele frequencies were found between MS patients and healthy controls.

Oxidative modification of fibronectin in inflammation site

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Fibronectin – the extracellular matrix protein is oxidatively modified with oxygen reactive species (ROS) in inflammation site. Activated neutrophiles release the hypochlorite acid (HOCl) and chloramines as products of myeloperoxidase/H_2O_2/Cl^- system. These reactive chlorine species chlorinate in turn matrix proteins. The resulting changes of tertiary protein structure could be evaluated by monitoring the antigen/antibody complex formation.

The formation of the complexes between native/chlorinated fibronectin and IgG class antibodies were examined by means of ELISA with luminol chemiluminescence detection. The degree of fibronectin modification was monitored with spectroscopic methods. Since the oxidation leads to the fibronectin aggregation – the tryptophane contents in resulting aggregates were evaluated with Stern-Volmer approach (acrylamide quenching). Moreover, the aldehydes influence on the Ag/Ab complex formation was examined – since aldehydes are known products of amino acids N–chloramines deamination. Also the native and modified fibronectin adherence to the matrix proteins was monitored with use of HRP labeled anti–fibronectin antibodies.

The preliminary results suggest that chlorination impairs the Ab/Ag complex recognition but also prove that IgG bounded chlorinated fibronectin promotes IgG clusters formation. It was found also that mM concentration of the serine derived glycoaldehyde decreases the fibronectin/IgG recognition and the effect could be attributed to the IgG aggregates formation. We demonstrate also that HRP–labeled IgGs detect the collagen and fibrynogen adherent fibronectin in a dose dependent manner–details of the ELISA method are discussed.

5-Hydroxy-2-aminovaleric acid as a specific marker of protein oxidation in rheumatoid arthritis synovial fluid

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In subjects with rheumatoid arthritis (RA) oxidized low density lipoproteins (LDL) are supposed to serve as mediators for joint damage, further exacerbating the inflammatory process. To better understand mechanisms of LDL oxidation in RA a specific marker of oxidative modification of apolipoprotein (apo) B-100 proline and arginine residues, 5-hydroxy-2-aminovaleric acid (HAVA), had been measured in plasma and synovial fluid LDL subfractions (LDL_1, Svedberg units (S_f) 7–12 and LDL_2, S_f 0–7) by GC-MS. Paired knee synovial fluid and plasma samples were collected from 10 subjects with RA. Additionally, plasma samples were collected from 10 healthy controls. The LDL_1 HAVA content in plasma was not different between the groups (RA, 0.004 ± 0.001 *vs* controls, 0.004 ± 0.001 mol/mol apoB-100, $P = 0.748$). The LDL_2 HAVA content in plasma was significantly higher in RA (0.145 ± 0.051 *vs* 0.013 ± 0.002 mol/mol apoB-100, $P = 0.000$). Furthermore, synovial fluid LDL_1 and LDL_2 in RA contained elevated HAVA levels when compared with plasma concentrations (LDL_{1syn}, 0.023 ± 0.012 mol/mol apoB-100 ($P < 0.001$) and LDL_{2syn}, 0.434 ± 0.129 mol/mol apoB-100 ($P < 0.001$)). Results suggest that proline and arginine residues of apoB-100 are highly reactive toward oxygen radicals in both plasma and synovial fluid in RA. Furthermore, susceptibility of apoB-100 to oxidative modification increases along the lipoprotein metabolic cascade. Particularly small dense LDL_2 were prone to direct oxidation of apoB-100. Correlation between HAVA content in plasma and synovial fluid LDL_1 and LDL_2 in RA may allow the use of HAVA as a clinical marker of antioxidant barrier impairment in RA.

Polymer of a proline analogue reverses vascular collagen accumulation in established pulmonary hypertension in rats

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Vascular collagen accumulation contributes to development of hypoxic pulmonary hypertension (PH). We have shown that injections of a polymer of the proline analogue *cis*-4-hydroxy-L-proline (cHyp) in liposomes attenuated acute PH in rats (*AJRCCM* 1997; 155:1384). We now treated rats with established PH with a new polymer containing an increased "payload" of cHyp. cHyp was conjugated to a low Mw poly(ethylene glycol)-Lysine carrier {poly (PEG1000)-Lys-cHyp} to increase the % by wt of the analogue. Rats were exposed to 10% O_2 for 7 da to induce PH. On da 0, 7 and 14 after 7 da of hypoxia, animals were injected iv with cHyp polymer in liposomes (Hc) or bioinactive *trans*-Hyp polymer in liposomes (Ht). Air controls received tHyp polymer in liposomes (At). At 0 and 21 da, we measured mean right ventricular pressure (RVP) and hydroxyproline (Hyp) content in main pulmonary arteries. On da 0, RVP (mmHg) was 9 ± 1 and Hyp (μg/vessel) was 88 ± 6 in At. RVP and Hyp increased to 17 ± 1* and 139 ± 4*, respectively, in hypoxic animals (n = 4; *P < 0.05 vs. At). On da 21, RVPs were at 10 ± 1, Ht 24 ± 1*, Hc 15 ± 1*†; Hyps were at 91 ± 9, Ht 176 ± 15*, Hc 122 ± 1*† (n = 4; *P < 0.05 vs. At; †P < 0.05 vs. Ht). From da 0 to 21, RVP did not increase and Hyp decreased in the Hc group vs. Ht. We conclude that weekly injections of polymeric cHyp prevented progression of established hypoxic PH and reversed Hyp accumulation. Targeted delivery of antifibrotic polymers may prevent and reverse the progression of PH. (Support: PHS, Barbara Cornwall Foundation).

Metabolism/Nutrition

Metabolic engineering of glucosinolate profiles

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Glucosinolates are amino acid-derived natural plant products found throughout the Capparales order, which includes agriculturally important crops such as oilseed rape, *Brassica* vegetables and the model plant *Arabidopsis*. Glucosinolates and their degradation products have a wide range of biological activities, e.g. in plant defense as deterrents against insect and fungi and as attractants to insects that are specialized feeders in *Brassicaceae*.

The conversion of amino acids to oximes is a key step in glucosinolate biosynthesis. We have recently shown that cytochromes P450 belonging to the CYP79 family catalyze the conversion of aliphatic, aromatic as well as indole amino acids to the corresponding oximes.

CYP71E1 catalyzes the oxime-metabolizing step in the biosynthesis of the cyanogenic glucoside dhurrin. We have recently shown that the oxime-metabolizing enzyme in the glucosinolate biosynthetic pathway is a cytochrome P450 homologous to CYP71E1. The post-oxime enzymes in the glucosinolate pathway have high substrate-specificity for the functional groups, and low substrate-specificity for the side chain. Therefore, we have been able to metabolically engineer new glucosinolate profiles into Arabidopsis by altering the level of endogenous CYP79s and by introducing new CYP79s. The approach has great potential for design of "biotech crops" with improved pest resistance and increased nutritional value.

Hypercalcemia as a potential threat in the dietary treatment of maternal phenylketonuria

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Over 90% of infants born to mothers with blood phenylalanine (phe) concentrations above 1200 μmol/L exhibit evi-

dence of foetal dammage, low birth weight, microcephaly, dysmorphic facies, slow postnatal growth and development and long-term intellectual impairment. Keeping maternal phe concentrations below 250 μmol/L before conception and throughout pregnancy reduces significantly the risk of abnormalities in the offspring of women with phenylketonuria (PKU).

We describe a woman, 31 years old, who showed phe blood levels of 150–200 μmol/L under a strict diet (total protein content of 1.13 g/kg body weight/day with 0.54 g/kg natural proteins and 0.59 g/kg proteins provided by the aminoacid mixture PKU3 (Milupa, Germany); 1,655 Cal/day) at the beginning of her first pregnancy. The first weeks she developed vomiting which gradually increased in severity. At 8 weeks of pregnancy, she had diarrhea, severe bouts of vomiting and manifested a deficient nutritional status with intake of 0.2 g/kg BW proteins and 1,178 Cal/day. She was hospitalized to start refeeding using continue drip feeding administered by nasogastric tube.

After 2 days on this regimen she developed vomiting, heart palpitations and mental confusion. Her serum calcium level, that was normal at admission in the hospital, showed an elevation to 6.5–7 mEq/L (ref. value 4.2–5.1 mEq/L). The feeding was stopped immediately and under an intravenous infusion and gradually introducing a feeding composed of PKU3, carbohydrates and MCT fats the serum calcium and the blood phe levels dropped to normal values.

	Administered	PKU3 (80 g = 1, 2 g/kg/BW)	ADH
Calcium (mg)	2,536	1,048	800–110
phosphate (mg)	1,613	808	700–1,600
magnesium (mg)	706	432	250–350
vit D2 (μg)	–	9.6	5–10

The hypercalcemia in this patient was due to a very high content in calcium of the feeding administered (2–3 times the ADH value) associated with a high vitamine D concentration (see table) and a clinical state of dehydratation.

The further pregnancy was uncomplicated and a healthy girl was born who developed normal.

Conclusions:

- The aminoacid mixtures used in the treatment of PKU contain a high level of calcium, phosphate, magnesium and iron. They also contain a high concentration of vitamine D.
- Nutritional monitoring of pregnant PKU patients should include the calcium, phosphate, iron, zinc and vitamins status.
- Vitamins A and D suppletion is contraindicated in these patients based on the high concentrations of these vitamins in the aminoacid mixtures used in the dietary treatment.

Catalytic effect of lysine in caramelization reaction

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Caramelization of various carbohydrates leads to product with a high tinctorial strength provided by different additives catalyzing the process. The present study was conducted to evaluate the catalytic effect of lysine on the sensory attributes and volatile components of caramel obtained by heating commercial maltose solution for different time intervals. One sample containing maltose only was used as control, the caramelization was conducted at 130c° for total time period 90 minutes and subjected to sensory analysis and isolation of volatile components. The odour and colour sensory tests were evaluated according to the international standard methods (ISO). The results showed that addition of lysine as a catalyst gave rise to a significant ($P < 0.05$) increase in intensity of the whole flavour in comparison with the control sample. The sweet and caramel notes, the most characteristic attributes of caramel, showed remarkable increase. On the other hand the increase in heating time in presence of lysine as a catalyst resulted in high significant increase in browning rate of caramel solution. The volatile components of each sample were isolated by using the new technique, solid phase microextraction (SPME) and subjected to GC and GC-MS analysis. Over 250 volatile components were separated, however only the most important component for caramel flavour were reported. Maltol and 5-hydroxymethyl-2-furfural (HMF) and 4. H-pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl (dihydro dihydroxy maltol), the main characteristic caramelization products were present in high concentration in samples contaning lysine heated for 60 minutes. In addition one pyrazine was only identified in the samples contaning lysine.

A comparative study between the present results and those of our previous study concerning addition of alanine as a catalyst was carried out.

Stimulation of L-arginine transport and eNOS by acute hyperglycemia requires phosphatidylinositol 3-kinase activity in human endothelium

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Short-term exposure of human umbilical vein endothelial cells (HUVECs) to hyperglycemia increases L-arginine transport (system y^+/CATs) and nitric oxide (NO) production (via eNOS). It has been reported that eNOS could also be activated by a Ca^{2+}-independent mechanism involving phosphorylation of Ser^{1177} by a phosphatidylinositol 3-kinase (PI3-kinase) dependent pathway. We investigated the involvement of PI3-kinase on the stimulatory effect of acute hyperglycemia on eNOS and L-arginine transport in HUVECs. L-Arginine transport, NO synthesis and phosphorylation of Ser^{1177} in eNOS were increased by D-glucose (25 mM, 2 min). Similar results were obtained in HUVECs exposed to insulin. Incubation of cells with wortmannin (PI3-kinase inhibitor) prevented the effects of D-glucose and insulin. No changes in the intracellular Ca^{2+} and eNOS protein levels were detected. Thus, acute hyperglycemia increases L-arginine transport and eNOS activity through a PI3-kinase dependent, Ca^{2+} independent mechanism in HUVECs.

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Protein lipoylation: characterization of lipoate-activating enzyme

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Lipoic acid is a prosthetic group of H-protein of the glycine cleavage system and E2 components of the pyruvate, 2-oxoglutarate and branched-chain 2-oxoacid dehydrogenase complexes. In mammals, attachment of lipoic acid to these proteins requires two enzymes. Lipoate-activating enzyme (LAE) catalyzes the activation of lipoate to lipoyl-nucleoside monophosphate. Then, lipoyltransferase transfers the lipoyl moiety to the specific lysine residue of the proteins. We purified LAE from bovine liver mitochondria. LAE activated lipoate with GTP at a 1000-fold higher rate than with ATP. The reaction absolutely required lipoate and MgGTP, and the reaction product was lipoyl-GMP. LAE activated both R- and S-enantiomers of lipoate to the respective lipoyl-GMP although preference for R-lipoate was observed. Lipoyltransferase equally transferred both R- and S-lipoyl moiety from respective activated lipoate to apoH-protein. However, only H-protein carrying R-lipoate was active in the glycine cleavage reaction. cDNA clones encoding a precursor LAE with a mitochondrial presequence were isolated. Amino acid sequence of LAE was identical with that of xenobiotic-metabolizing/medium-chain fatty acid : CoA ligase-III, but an amino acid substitution due to SNP was found. These results indicate that the medium-chain acyl-CoA synthetase in mitochondria plays a novel function with GTP, the activation of lipoate.

NO-related control of the glycolysis: the role of glutathione and protein S-nitrosylation

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Nitric oxide (NO) can modulate red blood cells (RBC) glycolysis by translocation of the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPD) [E.C. 1.2.1.12] from the cytosolic domain of the membrane protein band 3 (cdb3) in the cytosol. In this study we have investigated which NO-reactive thiols might be involved influencing GAPD translocation, and which is the role of glutathione (GSH) in this context. Two highly reactive Cys residues (K_2 = 73.7 $M^{-1}s^{-1}$ and 101.5 $M^{-1}s^{-1}$, respectively) were identified by transnitrosylation with nitrosoglutathione (GSNO) of cdb3 and GAPD. The Cys 149 in the catalytic site of GAPD is exclusively involved in this GSNO-induced nitrosylation. Reassociation experiments carried out at equilibrium with preparations of RBC membranes and GAPD revealed that different NO-donors may form −SNO on, and decrease the affinity between, GAPD and cdb3. In intact RBC, both the NO-donors 3-morpholino-sydnonimine (SIN-1) and peroxynitrite ($ONOO^-$) significantly increased GAPD activity in the cytosol and glycolysis measured as lactate production and energy charge levels. However, we obtained data suggesting that $ONOO^-$ is the main NO-derivative able to cross the RBC membrane leading to GAPD translocation and −SNO formation. Both in cell-free experiments and intact RBC, diamide (a thiol oxidant able to inhibit GAPD activity) was observed to reverse the effect of SIN-1 on GAPD translocation. The results demonstrate that cdb3 and GAPD contain reactive thiols that can be transnitrosylation mainly by means of GSNO, these can ultimately influence GAPD translocation/activity and the glycolytic flux.

Do we still need a hypocaloric parenteral nutrition in surgical patients?

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New surgical procedures like minimal-invasive-surgery brought many advantages for the surgical patient: Less pain and shorter hospitalization.

Regarding nutrition, patients gets normal food on the ward still on the operation-day and need only saline-infusions overnight for fluid and electrolyte substitution but no hypocaloric parenteral nutrition.

Hypocaloric parenteral nutrition had been developped as a peripheral intravenous nutritional concept for patients with a normal body mass index over a period not longer than 4–5 days.

Multiple clinical studies showed that bowel movements increase earlier after an early postoperative enteral feeding which allows an earlier discharge of the patient. The result is a remarkable decrease of costs and an increase in patient benefit.

Still some years before surgeons preferred in visceral surgery parenteral nutrition over a period of 4–5 days under the opinion not to stress an anastomosis. This opinion changed in the last years under the aspect that about 1,000–1,500 ml of bile fluid, 1,000–1,500 ml pancreatic juice and 1,000–1,500 ml gastric juice per day are passing a small intestine anastomosis without any complications. Concerning colon-anastomoses, the colon is preoperatively washed out, so it lasts until 5 days until defecation.

Multiple studies also showed a benefit for the patient regarding immunostimulation by early postoperative enteral feeding.

Conclusion: In our hospital with 244 surgical patients we recommend postoperatively either early normal enteral feeding or a high caloric parenteral nutrition if parenteral nutrition is needed for longer than 5 days. If artificial nutrition is necessary for more than 14 days we recommend enteral nutrition given by a tube or PEG (percutaneous endoscopic gastrotomy).

Study on the mechanization of some products of wheat flour

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In the Near East, "Frekeh" has been known for many centuries as a stable food made from wheat. It is generally claimed that "Frekeh" is better than wheat regarding its storage stability.

The protein quality of parched immature durum wheat (Frekeh) produced from 2 variety was evaluated. Frekeh from four maturing levels during the dough stage of the seed development, were analyzed for approximate analysis.

Results showed that "Frekeh" produced at the beginning of the dough stage was of better nutritional value than that produced at the following maturity levels, since the former was higher in protein, fat, minerals and crude fiber as well as in reducing sugar content. In addition, it was shown that these results confirm well with the sensory quality evaluation of the cooked product. Further more, it was found that the cooking time was suitable to produce a "freqkeh" meal with high levels of acceptability.

The observed decrease in protein content with increasing maturity level raised the question of how the protein quality of "frekeh" versus that of nature wheat grains varied. In this investigation, the amino acid of "frekeh" was determined.

Acylcarnitine measurement for monitoring treated patients with propionic acidemia (PPA) and methylmalonic acidemia (MMA)

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Dietary treatment and carnitine supplementation has greatly improved long-term outcome of patients with PPA and (vitamin B12 unresponsive) MMA. However, metabolic decompensation may be frequent and final outcome in most patients show various handicaps. To investigate the usefulness of measuring free carnitine and acylcarnitines in dried blood by tandem mass spectrometry, we investigated 9 patients with PPA and 5 with MMA in a period of 8 months by weekly capillary blood punctures performed by the parents. Age of the patients were from 0.5 until 18 years. Clinical status at the time of blood drawing was evaluated by regular phone calls. Free carnitine in all patients substituted by oral carnitine treatment (50–100 mg/kg/day BW) was normal. The parameter best reflecting clinical status was the C_3/C_{16}-acylcarnitine quotient. Mean value in MMA and PPA patients showed a range of 11.5–29.4 (normal 1.5 +/− 0.36, n = 18), there was no difference between PPA and MMA patients. Individual mean values of the patients significantly increased when the patient was ranked higher in the clinical score system or during decompensation. Since measurement of acylcarnitines in dried blood by tandem mass spectrometry is easy to perform, this method may be used for home monitoring of patients with MMA and PPA.

Influence of acute treatment with 1,2,3,4-tetrahydroisoquinoline on the levels of glutathione and reactive oxygen species, and on the enzymatic activity of γ-glutamyl transpeptidase in dopaminergic structures of rat brain

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1,2,3,4-tetrahydroisoquinoline (TIQ) and its derivatives generated considerable interest as molecular species that may be implicated in the pathogenesis of Parkinson's disease (PD). In PD, apart from the lack of dopamine in the striatum, a decreased concentration of glutathione (GSH) is found in the substantia nigra (SN). It is also known that GSH depletion potentiates the toxicity of MPTP and 6-hydroxydopamine. However, there are no data available on the TIQ influence on GSH metabolism. The aim of the present study was to exemine the effect of acute TIQ administration on the levels of GSH and reactive oxygen species (ROS), and on the enzymatic activity of γ-glutamyl transpeptidase (γ-GT) in dopaminergic structures of rat brain. The investigation was carried out 4 h after a single dose of TIQ (100 mg/kg i.p.). At that time, a marked increase in the tissue GSH level and simultaneous significant inhibition of γ-GT were found in the structures studied. In TIQ-treated rats, the production of ROS was reduced in the SN, but it was markedly enhanced in the striatum. Our results suggest that the increase in GSH level in dopaminergic structures stems from inhibition of γ-GT and refers to the extracellular pool of this peptide. Apparently, the TIQ-mediated alterations in the levels of GSH and ROS may have some implications for the etiology of PD.

Tetrahydrobiopterin-responsive phenylalanine-hydroxylase deficiency with mutations distant from the tetrahydrobiopterin binding site

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Phenylalanine hydroxylase (E.C. 1.14.16.1) catalyzes the hydroxylation of phenylalanine to tyrosine in the presence of oxygen and the cofactor (6*R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin (BH_4). Mutations in the phenylalanine hydroxylase gene may cause phenylketonuria or hyperphenylalaninemia. Alternatively, disorders in BH_4-metabolism also result in an increase in phenylalanine concentrations but simultaneously affect other BH_4-dependent enzymes, consequently, causing a severe neurological disorder. Recently, several patients with a phenylalanine-hydroxylase deficiency but with normal BH_4-metabolism were reported who showed a significant decrease in blood phenylalanine concentrations upon treatment with BH_4. Indeed, two such patients in our hospital were also sensitive to daily oral doses of 5–10 mg BH_4/kg. The subsequent molecular genetic analysis revealed that patient 1 was homozygous for the widespread mutation Y414C and patient 2 was compound heterozygous for the mutations A104D and K320N. It is striking, that all mutations are located distant from the known BH_4-binding site and thus, should not be associated with BH_4-sensitivity. Additionally, further patients who share the same genotype are not sensitive to BH_4. Therefore, it must be concluded that factors independent of the phenylalanine hydroxylase gene, like e.g. individual chaperone proteins, influence the three-dimensional structure of the enzyme and thereby, enhance enzymatic activity in the presence of elevated concentrations of BH_4.

Phosphorilation of gag-like protein encoded by first open reading frame of retrotrasposon gypsy (mdg4) affects to interaction with nucleic acid

M. Malikowa

Retrotransposons are structurally similar to retroviral gag and pol which are required for their replication via reverse transcription, and seem to be an ancestral form of specialized retroviruses. Reverse transcription of retrotransposons was assumed to occur in virus-like particles as well as in retroviruses. RNA-packaging in this particles suggests a possibility of infection. Presumably, the formation of functional virus-like particles requires the interaction of gypsy RNA with a protein encoded by gypsy first open reading frame (ORF1) or a product of its processing. The objective of this work was to study whether the protein by this frame can bind with nucleic acids similarly to retroviral GAG-protein and how phosphorilation of that protein may influence to this interaction. Then gypsy ORF1 was cloned and expressed in Escherichia coli, and its protein product was purified by ion-exchange chromatography on DEAE-cellulose and affinity chromatography on heparin-Sepharose and tested electrophoretically. It was shown that recombinant protein bound with its own mRNA and with DNA. The affinity for ssDNA bing higher than for dsDNA. The binding constant was estimated with RNA.

The method utilizes the ability of nitrocellulose to bind proteins but not nucleic acids. Binding of 50% gypsy RNA was achieved with about 100 ng of the protein in 50 ml of the reaction mixture. The binding constant was 5× 108 M-1, which is consistent.

The structure of the putative nucleic acid-binding domain suggests that the protein is more similar to the core proteins of

spumaviruses of the family Retroviridae that to those of other retroviruses. Phosphorilation of gag-like protein encoded by first open reading frame of retrotrasposon gypsy (mdg4) affects to interaction with nucleic acid.

Disorders of metabolism of tryptophan, methods and first results of screening

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Tryptophan (Trp) in humans is catabolized by several pathways leading to various metabolites of kynurenine and indolic compounds formation. A number of diseases are connected with abnormalities in its excretion, but relationship of cause and effect is usually unclear. We introduced a two-step procedure for the detection of defects in metabolism of Trp: 1) TLC is employed when starting the investigation, 2) two HPLC methods were proposed and used at the next step, when pathological findings are to be proved and the individual metabolites quantified. The first HPLC procedure enables the assessment of tryptophan, indolylacry-loylglycine (IAG) and other five indolic compounds. The second method is intended to the monitoring of kynurenine and seven of its catabolites. The same Sep-Pack pre-treated sample of plasma and urine is used for all methods. The reference values and the excretion pattern in some groups of patients (350 in total) were assessed. Hepathopathy, gastrointestinal defects, myopathy and seizures with other neurological symptoms were the conditions connected with changes in the excretion of some metabolites of Trp. Significant decrease of IAG excretion was found in burn patients early after the injury. Urine analyses were performed at patient with Hartnup disease and benign xanthurenic aciduria, inherited metabolic defects of Trp. In other experiments, Trp effect on the decarboxylation of other aromatic amino acids in the liver was investigated; only weak inhibition under physiological conditions was recognised.

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Characterization of D-threonine dehydrodegenase homologues of Escherichia coli

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Two hypothetical proteins of *Escherichia coli*, YbbQ and YhaE, show high sequence similarity to D-threonine dehydrogenase from *Pseudomonas cruciviae* IFO 12047. We cloned each gene encoding YbbQ and YhaE into *E. coli* JM109. Both YbbQ and YhaE showed no D-threonine dehydrogenase activity and showed significant activities for D-serine in the presence of NAD. YbbQ and YhaE were purified to homogeneity from the *E. coli* clones. YbbQ consisted of two identical subunits with a molecular mass of 31 kDa, whereas YhaE was a tetramer (native molecular mass, 124 kDa). YbbQ showed the maximum activity at pH 11.0 for the oxidation of D-serine. Whereas optimum pH of YhaE was pH 10.5. They catalyzed oxidation of glycerate and 3-hydroxyisobutyrate. D-Glycerate was the best substrate for both enzymes. Both enzymes also catalyzed reduction of tartronate semialdehyde in the presence of NADH. At physiological pH, the rate of tartronate semialdehyde reduction was much higher than that of D-glycerate oxidation. The *ybbQ* gene is in the operon of glyoxylate utilization and the *yhaE* gene is in the operon for D-glucarate/galactarate utilization. These results suggest that both YbbQ and YhaE are D-glycerate 3-dehydrogenases and function physiologically in conversion of tartronate semialdehyde into D-glycerate.

A serine protease inhibitor model: Synthesis and biology

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SGCI is structurally related to the PMPD-2 family of canonical serine protease inhibitors. In these peptides, there is a P1–P1′ position which is responsible for reversible binding to chymotrypsin. Their structure is characterized by structural compactness: the molecule contains three β-sheets and three disulfide bonds. In the SGCI molecule the P1–P1′ corresponds to Lys–Leu bond, which is cleaved by chymotrypsin extremely slowly. The question arises why an excellent substrate behaves at the same time as inhibitor. It was assumed that the three-dimensional structure of the molecule is responsible for the inhibitory activity.

A model was designed to include all the known features of the inhibitor: the structurally necessary β-sheet structure and the fragment containing the P1–P1′ environment.

Three model peptide were synthesized. Two model peptides had no inhibiting effect and were cleaved by chymotrypsin. One of the cleavage points is the expected P1–P1′ position, while the other positions found to be chymotrypsin preferred positions after the first cleavage.

The three-dimensional structures of the model peptides were mapped by NMR. On the basis of NMR structures obtained it has been shown that the cyclopeptide part is more flexible in the models than in SGCI.

The initial process in the reaction mechanism of a bisubstrate enzyme, rat mercaptopyruvate sulfurttransferase: Inactivation study by using chloropyruvate

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To investigate the reaction mechanism of a bisubstrate enzyme, rat mercaptopyruvate sulfurtransferase (EC 2.8.1.2, MST), inactivation kinetics with 3-chloropyruvate (chloropyruvate) was studied; each inactivation reaction was completed in a preincubation procedure. Chloropyruvate is an analog of 3-mercaptopyruvate (mercaptopyruvate) and irreversibly inhibits MST. The inactivation depended on incubation time and the concentration of chloropyruvate and showed saturation kinetics. The plot for the logarithm of % activity remaining versus preincubation time showed pseudo-first-order. The *kinact* is $8.0 \times 10^{-2}\,\mathrm{min}^{-1}$ and K1 is 3.1 mM. These suggest that chloropyruvate serves as a mechanism-based inactivator. Mercaptoethanol, an acceptor substrate, protected the inactivation. On the other hand, thiosulfate, a donor substrate, facilitated the inactivation. Mercaptopyruvate facilitated the inactivation as a donor substrate when

[chloropyruvate] > [mercaptopyruvate]. On the contrary, mercaptopyruvate protected it as an acceptor substrate when [chloropyruvate] < [mercaptopyruvate], so that chloropyruvate can approach Cys247 via the donor substrate route and acceptor substrate one, and a ternary complex may be formed prior to the inactivation. These findings suggest that a donor substrate enters the catalytic cavity prior to an acceptor one in the initial process of the MST reaction: MST follows an ordered sequential mechanism.

Stoichiometric analysis of the minimal actinorhodin polyketide synthase system

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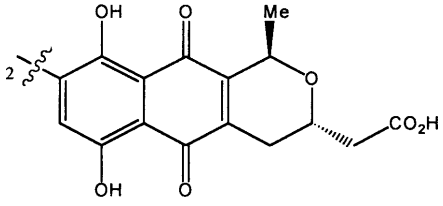
Polyketides are natural products of bacteria, fungi, marine organisms and higher plants, many of which have clinical usage. Actinorhodin **(1)** is an antibiotic produced by *Streptomyces coelicolor via* an iterative Type II polyketide synthase (PKS) system. This consists of a multi-enzyme complex with a single catalytic function for each enzyme.



(1)

The minimal actinorhodin PKS, shown in black below, consists of the ketosynthase (KS), chain length factor (CLF) and acyl carrier protein (ACP) and is the minimum set of enzymes required for polyketide production.

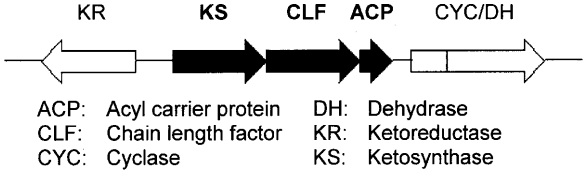


Fig. 1. Actinorhodin Gene Cluster

We have investigated the stoichiometry of the KS-CLF complex and the KS-CLF:ACP minimal system using three methods:

1. Native gel electrophoresis.
2. Cross-linking of proteins using dibromoacetone.
3. Radical cross-linking of proteins. This new method has also been used with wild type *S. coelicolor* cell free extract with 10% KS-CLF in order to elucidate which proteins are in close proximity to KS-CLF during *in vitro* actinorhodin production.

Protective and preventive effect of melatonin on acute liver injury in rats intoxicated with α-naphthylisothiocyanate

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It has been reported that, in rats with a single intoxication of α-naphthylisothiocyanate (ANIT), acute liver injury develops with enhanced lipid peroxidation and neutrophil infiltration in the liver tissue. Melatonin functions as an antioxidant. Melatonin is known to inhibit neutrophil infiltration into damaged liver tissues. Therefore, we examined whether melatonin exerts a protective or preventive effect on ANIT-induced acute liver injury. Male Wistar rats received a single i.p. injection of ANIT (75 mg/kg) and oral administraton of melatonin (100 mg/kg) at 12 or 24 h after ANIT injection. Animals administered with melatonin at 12 and 24 h after ANIT injection were sacrificed 24 and 48 h, respectively, after the injection. Liver injury appeared 24 h after ANIT injection and developed at 48 h. Melatonin administered at 12 h after ANIT injection prevented liver injury formation with attenuation of increases in hepatic lipid peroxide level and myeloperoxidase activity, an index of neutrophil infiltration. Melatonin administered at 24 h after ANIT injection prevented liver injury development with attenuation of further increase in hepatic lipid peroxide level. Thus, melatonin protects against and prevents ANIT-induced acute liver injury in rats possibly through its antioxidant action and/or its inhibitory action against neutrophil infiltration in the liver tissue.

Expression and characterization of human T-protein of the glycine cleavage system with mutation identified in nonketotic hyperglycinemia

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T-protein is a component of the glycine cleavage system and catalyzes the tetrahydrofolate-dependent reaction. Mutation in human T-protein (HT) gene results in clinical nonketotic hyperglycinemia (NKH). Eight point mutations have been identified so far in NKH patients with T-protein deficiency. To understand the structure and function of HT, the wild-type (wtHT) and three mutant T-proteins (G47R, G269D and R320H) were expressed in Escherichia coli with chaperons GroEL and GroES which facilitated the recovery of the expressed proteins as a soluble form. Levels of expression of these proteins were similar but the recovered soluble forms of mutants were about one-third of wtHT. G47R showed comparable specific activity to wtHT, whereas G269D and R320H mutants exhibited remarkable reduction in specific activity. Since homoallelism for G269D mutation and heteroallelism for G47R and R320H mutation were identified in typical and atypical NKH, respectively, these results suggest that G269 and R320H mutations are highly deleterious in the aspects of not only protein folding and/or stability but also catalytsis. On the other hand, G47R mutation might affect mainly on the protein stability. Detailed characterization of these mutants is now in progress.

Is histidine essential for ruminant animals?

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In ruminant animals, essential amino acids have never been completely established, because of the difficulty of its estimation due to the presence of microorganisms such as bacteria and protozoa in the first stomach called rumen. In our previous paper, histidine was shown to be the first limiting amino acid in the rumen contents when evaluated by chemical score. Recently we have also reported that rumen microorganisms cannot synthesize histidine from histidinol. On the other hand, there have been some reports which showed that nitrogen balance of ruminants was not improved by supplementation of histidine to rumen microbial protein together with methionine, lysine and threonine which had been known to improve. Based on these facts, we have a hypothesis that histidine may not be an essential amino acid for ruminants. In the present paper, we will report about the abilities of cattle liver and kidney to synthesize histidine from histidinol comparing with those of swine liver and kidney. The ability was demonstrated by examining the activities of histidinol dehydrogenase (crude enzyme) by means of direct measurement of an increase in histidine and decrease in histidinol. The amount of histidine produced from histidinol by the enzyme seemed sufficient for meeting the histidine requirement of cattle.

Conditioning of amino acids-sugar nonenzymatic interaction in flavour production

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The browning reaction is the sequence of events which begins with the reaction of amino group in amino acids, peptides or proteins with glycosidic hydroxyl group of sugars; the sequence terminates with the formation of brown nitrogenous compounds or melanoidines. This reaction gives rise to tremendous number of components such as volatile alcohols, ketones, aldehydes, esters, ethers and sulfur and nitrogen containing heterocycles in addition to nonvolatile Amadori compounds and complex brown pigments of medium to high molecular weights.

The present study was designed to choose a currently occurring system (aspartic acid – fructose) as a model system, since aspartic acid was found to be one of the most important amino acids in many kinds of food varieties. The reaction was done under controlled conditions of reactants ratios, temperature and time. The reaction mixtures were subjected to successive extractions with suitable solvents where the obtained corresponding flavour concentrates were thoroughly investigated. The results indicated different classes of compounds such as aldehydes, furans, alcohols and alkylated pyrazines varying in quantities depending on the reaction conditions. These products were also investigated concerning their toxicological effects. So, such products of nonenzymatic reactions showed different chemical and biological properties.

Alanine dehydrogenase from *Aeromonas hydrophila*: Purification and characterization

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NAD^+ dependent alanine dehydrogenase was purified 100 fold to homogeneity from *Aeromonas hydrophila*. Molecular mass of 230,000 daltons was estimated for alanine dehydrogenase by Sephadex G-200 chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified enzyme showed 1 polypeptide band with molecular mass of 40,000 daltons, indicating that the enzyme is hexamer. The enzyme is highly specific for alanine and NAD^+. Sulfhydryl group of the enzyme plays an important role in the catalysis. The enzyme retained its activity on heating at 55°C for 16 h. Optimum pH for reductive amination and oxidative deamination were 8.0 and 10.5, respectively. The steady state kinetic studies including product inhibition on the enzyme reaction indicated that the oxidative deamination proceeds through a sequential ordered binary-ternary mechanism in which NAD^+ binds first to the enzyme followed by L-alanine and products are released in the order of pyruvate, ammonia and NADH, respectively. The K_m values for NAD^+, L-alanine, pyruvate, ammonia and NADH were 0.17, 20, 1.33, 77 and 0.25 mM, respectively.

The metabolic basis of VLDL-overproduction: Insights from stable isotope studies

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An elevation of apolipoprotein (apo) B-100 concentrations is a particular feature of several metabolic disorders, such as type 2 diabetes (T2D), impaired glucose tolerance (IGT), and familial combined hyperlipidemia (FCHL). To further understand the *in vivo* turnover of apolipoprotein B-100 of very low density lipoprotein subfractions ($VLDL_1$, Svedberg units (S_f) 60–400 and $VLDL_2$, S_f 20–60) kinetic studies were performed in subjects with T2D, IGT, FCHL, and healthy controls using a tracer of either L-[ring-$^{13}C_6$]-phenylalanine or L-[5,5,5-$^2H_3$]-leucine. These studies showed direct hepatic $VLDL_1$ apoB-100 secretion to be increased in patients with T2D and IGT when compared with controls. In contrast, patients with FCHL showed a discrete increase in hepatic $VLDL_2$ apoB-100 secretion. In all patients VLDL catabolism is not essentially impaired. $VLDL_1$ apoB-100 secretion is associated with plasma insulin and free fatty acid (FFA) concentrations, resp., whereas $VLDL_2$ apoB-100 secretion is correlated with plasma mevalonate and lathosterol levels. In conclusion, VLDL overproduction is supposed to be completely responsible for higher triglyceride (TG) levels found in patients with T2D, IGT, and FCHL. $VLDL_1$ overproduction seems to be regulated by TG and FFA substrate and appears to be an indicator of decreased insulin sensitivity. In contrast, $VLDL_2$ overproduction is more likely to be regulated by the availability of cholesterol substrate. These data give further *in vivo* evidence that $VLDL_1$ and $VLDL_2$ secretion is regulated independently.

Overexpression of mutated serine acetyltransferase gene in *Arabidopsis* resulted in enhanced production of cysteine and glutathione

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Serine acetyltransferase (SATase) catalyzes the formation of *O*-acetyl-L-serine (OAS) which is the key intermediate of cysteine biosynthesis. OAS is not only a dominant limiting factor but recently suggested as a possible signal molecule for gene expression in cysteine biosynthesis. In has been shown that the activity of cytosolic SATase from watermelon was feedback inhibited by L-cysteine. To enhance the ability of cysteine biosynthesis in plants and to reveal the role of OAS in the regulation of sulfur assimilation, we made the point-mutated watermelon SATase gene (SATG277C) whose product was not inhibited by cysteine, and introduced SATG227C into

Arabidopsis. The contents of OAS, cysteine, and glutathione in transgenic *Arabidopsis* were increased significantly as compared to the wild-type *Arabidopsis*. We are currently dealing with the expression analysis of sulfur-related genes in transgenic *Arabidopsis* accumulating OAS due to the overexpression of SATase.

Certain amino acids as source of specific branched chain fatty acids in fish sauce manufacture

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The source of some branched volatile fatty acids (VFA) during the fermentation process in the manufacture of fish sauce was investigated. We previously reported that straight chain volatile acids seemed to have been derived from fish fats but unlikely for branched fatty acids which was believed to be derived from other sources. To clarify the source of branched volatile acids, specific amino acids, alanine, leucine, iso-leucine and valine were used in this study. These amino acids were first mixed with salt and added to fish. The fish mixtures were then aerobically and anaerobically incubated for one and a half months. Results showed that addition of valine significantly increased the production of iso-butyric and iso-hexanoic acids and leucine increased that of iso-valeric in the aerobically fermented fish mixtures. A similar tendency was observed in the anaerobically fermented fish mixture except that an increase in the amount of iso-hexanoic acid was observed in the leucine added mixture, which was not observed in the aerobically fermented one. It seemed that specific branched volatile fatty acids were derived from certain amino acids.

Glutathione transport in retinal Müller (glial) cells

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Glutathione (GSH) is an important component of the cellular defense mechanisms that protect cells from oxidative injury. In the retina, the glial (Müller) cells have been shown to synthesize and transport GSH, and thus are likely to be involved in regulating GSH levels. In the present study, we have characterized GSH transport system in a Müller cell line using ^{35}S-GSH uptake. The results showed that GSH was taken up in a Na^+- and concentration-dependent manner with a K_m of 0.31 mM. Moreover, Cellular GSH had no effect on the rate of GSH uptake. In related studies, we found that oxidative stress induced the expression of γ-glutamylcysteine synthetase (GCS) subunits, and that GCS mRNA levels were correlated with the degree of GSH depletion. Because organic anion transporters (oatps) have been implicated in glutathione co-transport, we examined expression of oatp members using RT-PCR. We found that the Müller cell line expressed transcripts for oatp1, oatp2 and oatp3. These studies indicate that the Müller cell plays important role in GSH homeostasis in the retina.

Characterization of H131R mutant porphobilinogen synthase cloned from Hep3B cell

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In the active site of human porphobilinogen synthase (EC 4.2.1.24, PBGS), two zinc ions are coordinated by Cys^{122}, Cys^{123} and Cys^{132}, and His^{131} and Cys^{223}, respectively. The fomer zinc ion, closer to catalytic site Lys^{252}, plays an important role in catalysis. On the other hand, a role of the latter (distal) one has not been clarified. Interestingly, in human Hep3B cell, His^{131} was replaced with Arg (H131R). To elucidate the role of His^{131} in catalysis, the kinetic properties of wild type and H131R mutant enzymes were studied. These cDNAs were cloned by RT-PCR with total RNA from human peripheral lymphocyte and Hep3B cell, respectively. Each cDNA encoding PBGS with 3′ non-coding region was inserted into pET-22b(+) vector and then the constract was transformed into *E. coli* strain BL21(DE3). The cells were cultured in LB medium containing 50 mg/ml ampicillin and 10 μM Zn ion for 3 h at 37°C. After addition of 1 mM isopropyl-β-D(−)-thiogalactopyranoside, cells were further cultured for 24 h at 20°C. The highly purified PBGSs were obtained by ultora centrifugion, fractionation with ammonium sulfate and column chromatographies with DEAE-cellulose, hydroxylapatite and Superdex 200, serially. We are now investigating molecular properties of these PBGSs.

The use of amino acids in attenuating HPA response in cervidae

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Handling and management procedures such as capture and restraint can be significant stressors for recently domesticated animals such as elk (Cervidae elaphus). The objective of the current study was to investigate the use of pre capture nutritional therapy in attenuating HPA response and improving animal welfare. Fourty eight adult male elk stags ranging in age from 2–4 years and raised on pasture were used in the study with 23 as control and 25 as nutritionally treated. Twenty four hours prior to capture the elk were offered either 1 kg of a cereal grain based dietary supplement or 1 kg of a cereal grain based nutritional therapy product containing specified amino acids (USA patent # 5505968). The amino acid content of the nutritional therapy product was minimally 0.5 g per 500 kg animal weight of ala, lys, phen, meth, thre, isoleu, val and tryp plus 15 g per 500 kg weight of leu and 40 g/500 kg weight of glut. The animals were subsequently captured and held in appropriate facilities designed to handle elk. Saliva samples were collected on all animals immediately following capture and salivary cortisol was monitored by RIA. Animals offered the nutritional therapy product containing the amino acid mixture displayed lower cortisol levels (11.8 nmol/L) compared to the untreated controls (14.9 nmol/L; $P < 0.05$). The data suggest that amino acid therapy can be used to attenuate HPA response to a stressor in captured elk.

Production of L-methionine by Coynebacterium mutants

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Resistance to analogues of methionine by *Corynebacterium lilium* results in the partial de-repression of methionine biosynthetic enzymes. The levels of enzymes involved in methionine biosynthesis also increased step-wise by successive endowing the resistant markers, resulting in the over-production of methionine. Moreover, the repressibilities of the enzymes were also reduced by the addition of methionine analogue resistance. Analogue resistant mutants were developed

by UV induced mutagenesis of *Corynebacterium lilium* (Wild Type) strain. The Single analogue (Norleucine) resistant mutant *C. lilium* NL-87 produced 372 µg/ml methionine in shake flasks with methionine yield at 0.068 g methionine/g glucose and specific methionine production at 0.237 mg/g DCW, while double analogue (Norleucine and Triazole) resistant mutant *C. lilium* NT-33 produced 521 µg/ml methionine. A triple analogue (Norleucine, Triazole and Ethionine) resistant mutant *C. lilium* NTE-99 produced 1.848 g/l methionine. The methionine yield was 0.248 g methionine/g glucose and its specific productivity was 1.04 g methionine/g DCW.

The effect of acetylcysteine on blood glucose in healthy and diabetic persons

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Blood plasma glucose level was compared on fast and 60 minutes after oral administration of 1200 mg of acetylcysteine.

In the group of 49 healthy persons the plasma glucose level feel by 7.6% over the 60 minute period.

In the 30 diabetics on the contrary, the plasma glucose level observed 60 minutes after administration of acetylcysteine was 11.8% higher than in blood plasma taken on fast.

Similar tests were carried out "in vitro" to interpret these different results. The control group consisted of 1 ml of distilled water +0.2 ml 10% glucose +0.2 ml GOD PAD (Boringer Mannheim GmbH).

In the acetylcysteine group the distilled water was replaced by 1 ml 0.01% solution of acetylcysteine. In the glucagon group the distilled water was replaced by 0.01% solution of GlucaGen hypokit Novo Nordisk.

Spectrometric determination was carried out after 60 minutes of incubation. A 27% diminution of glucose was observed in the acetylcysteine group in comparison with the control group. A 32.2% increase in glucose was observed in the glucagon group in relation to the controls.

The results with healthy persons and the tests "in vitro" indicate that acetylcysteine lowers the level of glucose. But it elevates the level of glucose in the blood plasma of diabetics. It may be presumed that acetylcysteine modifies the insulin-glucagon balance in favour of glucagon.

Fortification of yogurt with enzymatically modified oilseed proteins

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The objective of this study was to fortify Yogurt with three oilseed protein hydrolysates prepared from soybean (*Glycine max*), sesame (*Sesanum indicum*) and rice bran (*Oryza sativa*) flours. Hydrolysis was carried with two enzymes one of plant origin (Papain) and the other of microbial origin (Alcalase). A Yogurt fortification experiment was then carried using the previous hydrolysates. The hydrolysates were added to yoghurt at 5, 10 and 15% levels of fortification and the fortified yoghurt was analyzed fresh, and after 7 and 15 days of consuming period. Fortified Yogurt was chemically examined for fermentation activity (pH values, acidity and proteolysis) as well as its organoleptic properties. Results of this experiment indicate that the addition of soybean hydrolysates with Papain (8.9 Units/g) for 5 minutes (Tb) and rice bran hydrolysates with Alcalase (27.6 Units/g) for 5 minutes (Te) to yoghurt can exceed 5–10%, while fortification with sesame hydrolysed with Papain (8.9 Units/g) for 30 minutes (Td) and soybean hydrolysed with Papain (8.9 Units/g) for 30 minutes (Tc) can not reach up to 15%.

Acute hyperbaric oxygen (HBO) treatment rapidly up-regulate ODC and down-regulate SSAT in the rat heart, boosting polyamine tissue concentration: evidencies for a protective role of natural polyamines in heart failure as naturally occurring in vivo antioxidants?

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It is well known that DNA is fragile to reactive oxygen intermediates (ROIs) damage. Evidences that DNA fragmentation and apoptosis occur in cardiomyopathies, in the failing heart and in cultured cells under hyperbaric oxygen (HBO) stress, demonstrated that oxygen free radicals also play a critical role in heart failure. As a consequence, myocardial cell survival depends on response to oxidative stress. Experimental data obtained in vitro suggested that polyamines, by acting as ROIs scavengers, play a role in prevention of endonuclease-mediated DNA fragmentation and inhibition of alkylating agents-mediated damage, potentially exerting a protective role against ROIs damage. Thus we studied polyamine metabolism and superoxide dismutase (SOD) expression in an in vivo model of heart oxidative stress, such as rats subjected to HBO. Four experimental groups were used: 1) controls; 2) rats subjected to HBO for 15 min once and immediately sacrificed; 3) rats treated as group 2 but for 3 consecutive days and immediately sacrificed; 4) rats treated as group 3 but sacrificed 24 h later (recovery). Northern blot analyses showed that ODC mRNA accumulation increased immediately (paralleled by activity) in groups 2–4, while SSAT mRNA decreased remarkably, thus leading to higher polyamine concentration in ROIs-stressed hearts. Contrariwise, SOD mRNA level decreased rapidly in groups 2–4. This suggests that HBO-induced compensatory mechanism in rat heart is based on specific and rapid boosting of polyamine concentration, caused by coordinate induction of biosynthesis and inhibition of catabolism, and not of enzymes known to metabolise ROIs such as SOD.

Effects of leucine supplement diet on tumor development in pregnant rats

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Amino acids oxidation was greater in tumor-bearing rats muscle. Leucine is an important ketogenic amino acid that proves energy to the skeletal muscle. Leucine supplemented diet was used to analyze the effects produced by Walker 256 growing in pregnant rats which were distributed into six groups. Three groups received normal diet (18% protein): control (C), tumor-bearing (W), pair-fed rats (Cp). Three groups were fed with diet supplemented with 3% leucine (15% protein plus 3% leucine): pregnant fed with leucine (L), tumor-bearing with leucine (WL) and pair-fed with leucine (Lp). After 21 days, the animals were submitted to intestinal perfusion to measure leucine, methionine and glucose absorption. Leucine absorption increased in W and WL groups. Glucose absorption reduced in tumor-bearing. In pregnancy with cancer, metabolic changes provided both reduced fetal and tumor development.

Tumor-bearing rats showed increase in methionine and leucine absorption, probably diverting this nutrients to tumor cells. Glucose absorption reduced in W and WL. Leucine supplemented diet group promoted high leucine absorption which could be used by neoplasic cells, and mainly by fetus and host. Probably, the transamination of the branch long chain amino acid provided energy substract for the skeletal muscle, keeping the nitrogen offered to host carcass.

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The effects of nutritional replacement and leucine diet supplementation on rats chemical body composition

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Undernutrition cause several changes as body weight loss, in biochemical parameters, even microscopic alteration in absorptive epithelium. This means the nutrients absorption process has been harmfully and consequently increase the damages caused by malnourished. Knowing leucine is used as a ketonic and oxidative amino acid our main propose was to recovery the malnourished young rats with normal (RC) and leucine supplemented diet (RL, 3% of leucine) for 60 days. It was measured body, liver, and muscle weight, intestinal absorption of glucose, methionine and leucine, and body chemical composition. The body weight gain in RC and RL was higher than control group, suggesting that nutritional replacement for these groups could provided nutrients to support the body weight recovery, reaching as the same weight as the control. Methionine and glucose absorption was reduced in malnourished group, but it was recovered (glucose, methionine and leucine) after nutritional replacement. Leucine supplemented diet promoted a good recovery of carcass collagen nitrogen, keeping the carcass structural nitrogen. Further studies are necessary to investigate this mechanism.

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Interaction of the proline metabolite L-Δ^1-pyrroline-5-carboxylic acid with pyridoxal phosphate and other biologically important aldehydes and ketones

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We diagnosed the very rare autosomal recessive disorder Hyperprolinaemia type II (deficiency of Δ^1-pyrroline-5-carboxylate dehydrogenase, EC 1.5.1.12) in a girl aged 20 months presenting with seizures and encephalopathy. L-Δ^1-pyrroline-5-carboxylic acid accumulates in this disorder and there is a 10–15-fold increase in plasma proline. Surprisingly, she also had vitamin B_6 deficiency. This was an unrecognised association, which was not explained by her diet or medications. We hypothesised that pyridoxal phosphate (vitamin B_6 coenzyme) was de-activated by L-Δ^1-pyrroline-5-carboxylic acid.

With high resolution ^{1}H nuclear magnetic resonance spectroscopy and mass spectrometry, we have shown that these two compounds react at pH 7.4 and 37°C *in vitro* to form three novel adducts, which we characterised. They are products of a Claisen condensation (or Knoevenagel type of reaction) of the activated C4 carbon of the pyrroline ring with the aldehyde carbon of pyridoxal phosphate. If this previously unreported interaction occurs *in vivo*, pyrroline-5-carboxylic acid is a unique endogenous vitamin antagonist.

Preliminary observations show that pyrroline-5-carboxylic acid also condenses with other biologically important aldehydes and ketones. Some of these reactions may contribute to the brain disturbances in Hyperprolinaemia type II. We have already identified adducts with acetoacetic acid in urine from our child, which is evidence that condensation can occur *in vivo*.

The γ-glutamyltranspeptidase activity and nonprotein sulfhydryl compounds levels in rat kidney of different age groups

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The kidneys are characterized by a high activity of γ-glutamyl transpeptidase (γ-GT), as well as by a high cysteine level. The present paper was aimed to obtain information on how the activity of γ-GT and the levels of non-protein sulfhydryl compounds (NPSH) changed with age in rat kidneys. Simultaneously, protein-bound cysteine (Pb-Cys) and sulfane sulfur compounds were estimated. The kidneys were from following rats groups: young (3-month-old), middle-aged (19-month-old) and old (31-month-old). The obtained results showed that the activity of γGT and NPSH levels in the kidneys fell with age. At the same time, a significant increase in the level of protein-bound cysteine was observed. On the other hand, the content of sulfane sulfur compounds was elevated in the group of the oldest animals. These findings indicate that – due to disturbances in the γ-glutamyl cycle – the capacity for extracellular glutathione degradation and, in consequence, the availability of cysteine for intracellular GSH biosynthesis may be impaired. The increased Pb-Cys level indicates potentiation of the thiolation reaction, i.e. development of protein-mixed disulphides, cysteine, sulfane sulfur compounds, oxygen reactive species.

Study for importance effect of glycine B_{23} and phenylalanine B_{24} on insulin activity

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In the past few years, many attempts have been made to prepare a synthetic insulin. The biological activity of insulin is known to be closely related to the C-terminal octapeptide fragment of its B-chain. This does not necessarily mean, however, that each of the amino acid residues of the octapeptide fragment is essential for its activity. It was found that B_{23} gly and B_{24} Phe were present in all insulins so far obtained from various animal species indicating the significance of these two residues. It would therefore seem desirable to study the effect of each of these two amino acid residues or both on biological activity of the octapeptiede fragment of the B-chain. Weitzel et al. found that the substitution of arginine B_{22} with another amino acid resulted in a very large decrease in biological activity, which indicates that it participates in the action of insulin. Also it was found that the aromatic amino acid residues (B_{24}-B_{25}) participate in the action of insulin. A heptapeptide Arg-Phe-

Tyr-Thr-Pro-Lys-Ala-OCH_3, corresponding to (B_{22}-B_{30}) insulin des Gly_{23}-Phe_{24}, and an octapeptide Arg-Phe-Phe-Tyr-Thr-Pro-Lys-Ala-OCH_3, des Gly_{23} were synthesized using the solid phase method. The C-termenal ends of both peptide were converted to methyl ester by transesterification cleavage from the resin. The side chain protecting groups were removed by HF. Manual counter current distribution method was used for purification of the free peptides. The way to solve the evaluation of tyrosine containing peptide was studied. The free methyl ester peptides were administrated for insulin-like activity test by glucose metabolism in the rat fat cells technique *in vitro*.

Neurobiology

Nitric oxide synthase inhibitors influence dynorphin immunoreactivity in the rat brain following hyperthermia

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Nitric oxide (NO) is a free radical gas that influences neuronal communication in the central nervous system (CNS). Recent reports suggest that NO can influence dynorphin neurotransmission in the normal brain as well as in several pathological states. Previous reports from our laboratory show that the enzyme nitric oxide synthase (NOS) responsible for NO formation is upregulated in several brain regions following hyperthermia. The present investigation was carried out to find, whether hyperthermia can influence dynorphin immunoreactivity in the brain, and if so, whether inhibition of nitric oxide synthesis will alter its distribution in heat stressed rats. Rats subjected to hyperthermia at 38°C for 4 h in a biological oxygen demand incubator (BOD) resulted in marked redistribution of dynorphin immunoreactivity in several brain regions e.g., cerebral cortex, hippocampus, cerebellum and brain stem. Pretreatment with two potent NOS inhibitors, L-NAME (50 mg/kg, i.p.) and L-NMMA (30 mg/kg, i.p.) 30 min before heat stress significantly altered the dynorphin immunoreactivity in the brain. These drugs alone however, did not influence the peptide expression in normal rats. The results suggest that (i) hyperthermia has the capacity to influence dynorphin immunoreactivity in the brain, and (ii) inhibition of nitric oxide synthase considerably influences the dynorphin immunoreaction in hyperthermia, not reported earlier.

Acute and long-term functional alterations by NMDA antagonists

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The functional changes induced by uncompetitive and competitive NMDA antagonists, memantine, amantadine and MK-801, and CGP 40116, respectively, were studied in both saline-pretreated and MPTP-pretreated C57 BL/6 mice. The NMDA antagonists were administered acutely by themselves or in combinations of either: NMDA antagonist plus subthreshold L-Dopa dose or NMDA antagonist plus suprathreshold L-Dopa dose, to either the MPTP-pretreated or the saline-treated mice. Activity-enhancing or functional restorative effects of the NMDA antagonists were variable with memantine and MK-801 distinguished from amantadine and CGP 40116. In the study of long-term effect of NMDA antagonists MK-801 was administered postnatally and spontaneous motor behaviour and motor activity in response to several pharmacological interventions was assessed. Marked alterations associated possible with apoptotic penchance are discussed.

Restorative effects in experimental parkinsonism

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Synergistic antiparkinsonian actions of different classes of putative therapeutic agents co-administered with a subthreshold dose of L-Dopa (5 mg/kg) in drug-naive MPTP-treated mice as well as the restorative actions of those compounds in suprathreshold L-Dopa-tolerant MPTP-treated mice subjected to "wearing-off" of L-Dopa efficacy were assessed in a series of experiments. The classes of compounds studied included the noncompetitive NMDA antagonists, memantine, amantadine and MK-801, the anticonvulsive and putative anticonvulsive agents, lamotrigine, FCE 26743, phenytoin, the monoamine oxidase inhibitors, L-Deprenyl, amiflamine, α-ethyltryptamine, clorgyline and phenelzine, and the α_2-adrenoceptor agonists, clonidine and guanfacine. In this final case, the restorative effects of clonidine and guanfacine were antagonised by the α_2-adrenoceptor antagonist, yohimbine, but not the α_1-adrenoceptor antagonist, prazosin. Within each class of potentially therapeutic agents a differential restorative efficacy was obtained, but the combination of different doses of apomorphine with clondine failed to restore motor activity.

In vivo proton MR-spectroscopy of the human brain: Assessment of N-acetylaspartate (NAA) reduction as a marker for neurodegeneration

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Proton magnetic resonance spectroscopy (^{1}H-MRS) is a well accepted non-invasive method to investigate changes in brain metabolite composition in different types of cerebral disease. We performed proton spectroscopy in patients with dementia of the Alzheimer's type (AD) and in patients with motor neuron disease (MND) with the aim to detect a specific metabolic pattern for each of these two neurodegenerative disorders. Overall, more than 150 spectroscopic data sets of patients with MND and more than 100 data sets of AD patients were acquired within the last 5 years.

In the MND group we found a significant reduction of NAA/tCr metabolite ratios in the central region, which correlates with the disease severity and the clinical lateralisation of neurological symptoms and increases in the time course of the

disease. In AD patients a similar reduction in relative NAA contents was observed in the medial temporal lobe.

The observed regional metabolic alterations correlate well with the characteristic neurological symptoms in AD (dementia) and MND (muscular palsy) and seem to follow the disease process over time. Since NAA is exclusively expressed in neurons as shown by immunohistochemical studies, reduced NAA levels suggest neuronal loss or dysfunction in the observed regions.

Non-invasive imaging of angiogenesis *in vivo* using polypeptide-based probes

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Non-invasive measurement of hemodynamic parameters and imaging neovasculature architecture during angiogenesis is highly important in determining tumor prognosis and in assessing treatment efficacy. We suggested a technique to map the tumor vascular (VVF) and interstitial volume fraction (IVF) noninvasively *in vivo*. A poly-l-lysine based macromolecular probe (MPEG-PL-GdDTPA) with extended circulation in the bloodstream designed to shield chelated paramagnetic lantanide with poly(ethylene glycol) chains. We hypothesized that a magnetic resonance signal after intravenous administration of a vascular paramagnetic probe can be maximized so the signal change after administration of a second comound (GdDTPA) reflects the IVF but not the VVF. The method and its assumptions were verified in animal models of cancer. Tumoral VVF and IVF values were consistent with histology data and literature values. Imaging showed heterogeneity of both parameters at submillimeter pixel resolution. This technique was used for characterizing differential angiogenesis in human mammary adenocarcinoma lines as well as for imaging anti-angiogenic drug effects. Anti-angiogenesis was induced using synthetic D-reverse peptides derived from thrombospondin-1. This study showed that peptide treatment results in slower brain tumor growth due to inhibition of *de novo* blood vessel formation and synergistic anti-proliferative effect on tumor cells. In conclusion, *in vivo* MR imaging can be used for non-invasive treatment assessment of novel anti-angiogenic drugs.

Transcription factors involved in the pathogenesis of L-DOPA-induced dyskinesia in a rat model of Parkinson's disease

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We have recently found that 6-hydroxydopamine lesioned rats gradually develop dyskinetic- and dystonic-like movements upon repeated administration of a therapeutic dose of L-DOPA. Such movements simulate the time course of peak-dose dyskinesia in Parkinson's disease. In this rat model, the severity of L-DOPA-induced dyskinesia is strongly correlated with an upregulated expression of the prodynorphin gene in striatal neurons. Using antisense technology and gel-shift assay analyses, we have addressed the role of transcription factors which may mediate this response. We have found that the cAMP response-element binding protein (CREB) is essential in maintaining a basal expression of prodynorphin mRNA in the intact striatum, but it is not required for L-DOPA to induce the prodynorphin gene in dopamine-denervated striatal neurons. We have thus addressed the role of Fos- and Jun family transcription factors, and found very high levels of FosB- and JunD-like proteins in the striata of dyskinetic animals. These proteins could bind to both AP1 and CRE sites in the prodynorphin promoter. Moreover, intrastriatal fosB knockdown could inhibit both the upregulation of prodynorphin gene expression and the development of dyskinesias under chronic L-DOPA treatment. We propose that dimers of FosB- and JunD-like proteins mediate abnormal changes in striatal gene expression which are linked to the development of L-DOPA-induced dyskinesia.

Glutamatergic mechanisms in chronic pain

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Glutamate plays important roles in both normal and pathophysiological nocieception. Upon physiological conditions, glutamate release from primary afferents in the spinal cord activates largely AMPA receptors. As those are ubiquitously involved in fast transmission in the CNS, AMPA antagonists have a broad side-effect profile. Prolonged activation of nociceptors by tissue damage, inflammation or nerve injury evokes a long-lasting release of glutamate and neuropeptides, activating NMDA receptors in the spinal cord. This mechanism appears to play a key role in pain chronification. The NMDA receptor is, therefore, an important target for chronic pain treatment. Both animal and human studies confirm the efficacy of NMDA antagonists in chronic pain, however, clinically available compounds are weak or have unacceptable side-effects. $Glycine_B$ antagonists and compounds selectively blocking NR2B-containing receptors appear to be safer, the reasons for this remain unclear. Central side-effects could potentially be avoided by using NMDA antagonists with restricted central access. Peripheral NMDA receptors (as well as some other subtypes of GluRs) could be activated by glutamate released from the site of injury, thus contributing to peripheral hyperexcitability. Some other subtypes of GluRs can also contribute to peripheral sensitisation. Of ionotropic GluRs, kainate receptors appear important in inflammatory and neuropathic pain. They can be activated by high intensity stimulation of nociceptive afferents, and may act as autoreceptors controlling release of glutamate. Group I metabotropic GluRs are also present on primary afferents and on second order neurones in the spinal cord, and may play a similar role. Antagonists of these subtypes of GluRs are active in some models of chronic pain. Specific upregulation of group II metabotropic GluRs in some pain-relevant structures could reflect a possible adaptive role of these inhibitory receptors under chronic pain conditions; their selective agonists also have a potential for the treatment of chronic pain.

Physiological roles and regulation of metabotropic glutamate receptors in the basal ganglia

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We have performed a series of studies of the distribution and function of mGluR subtypes in the basal ganglia that suggest that members of this receptor family could serve as targets for novel therapeutic agents that would be effective in treatment of PD. For instance, two group II mGluRs (mGluR4 and mGluR7) are localized on presynaptic terminals of striatal neurons in the globus pallidus where they could reduce GABA release. Furthermore, activation of group I mGluRs results in a

depolarization and increased cell firing of neurons in the subthalamic nucleus (STN) and projection neurons of the substantia nigra pars reticulata (SNpr). Interestingly, this effect is mediated by mGluR1 in SNpr projection neurons and mGluR5 in STN neurons. Finally, activation of group II mGluRs results in inhibition of glutamate release from STN terminals in the SNpr. Furthermore, selective agonists of group II mGluRs inhibit haloperidol-induced catalepsy in rats, suggesting an antiparkinsonian effect of these compounds. The rich distribution and diverse physiological roles of mGluRs in basal ganglia raises the possibility that these receptors may provide targets for novel therapeutic agents that could be used for treatment of PD and related disorders.

Studies of the anoxic damage in rat hippocampal slices

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In vitro rat hippocampal slices are commonly used to study the effects of hypoxia in the central nervous system, because they allow to differentiate the effects of hypoxia in the brain from that of systemic (e.g., respiratory and cardiac) failure that may accompany hypoxia. We used electrophysiology to monitor and evaluate the damage caused by transient hypoxia to the nervous tissue. A few minutes after oxygen deprivation brain tissue suddenly depolarizes. This event, which is termed ìanoxic depolarizationî is accompanied by dramatic metabolic changes: transmembrane ionic gradients disappear (Na^+ enters, K^+ exits the neurons), neurons swell, there is intra- and extra-cellular acidosis. This event is caused by functional inactivation of (Na^+/K^+)ATPase caused by decreased ATP content, as it is suggested by the fact that it is mimicked by ouabain treatment. One of us has contributed to show that if this event is not promptly reversed by reoxygenation it causes irreversible damage, mainly by determining a massive entry of Ca^{2+} into neurons. Pretreatment of tissue with creatine (1 mM or more) both increases neuronal energy store by increasing neuronal phosphocreatine and protects brain tissue from irreversible damage. In vivo increase in phosphocreatine has been shown using lower (0.5 mM) creatine concentration, injected directly into the lateral ventricle. A different type of hypoxia-induced damage is observed when hypoxia is of shorter duration. In this case upon reoxygenation one does not observe disappearance of evoked potentials but their increase. This phenomenon, originally described as ìpost-hypoxic hyperexcitabilityî has been later called ìanoxic long-term potentiation (LTP)î. We showed that this event can be prevented by inactivating the nuclear protein S-100. While this damage is milder than that induced by anoxic depolarization, it may explain stroke-induced epileptic fits. We are currently investigating what role, if any, pretreatment with creatine may have in preventing also this type of damage.

Effects of cytoskeletal modification on Ca^{2+} influx in cerebral ischemia

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Cytoskeleton is subject to continuous modification to yield changes in cell shape and function of plasmamembrane proteins linked to the cytoskeleton. Gelsolin (gsn) depolymerizes filamentous actin and thus causes dynamic uncoupling of membrane ion channels. We have studied alteration of neuronal Ca^{2+} influx by the absence of gsn and its pathophysiological consequences during cerebral ischemia.

Cytosolic Ca^{2+} concentrations were determined ratiometrically in synaptosomes preloaded with fura-2AM. Glutamate release from synaptosomes superfused with Krebs' buffer was measured by HPLC. Transient focal cerebral ischemia was induced by 2 h occlusion of the middle cerebral artery (MCA).

In gsn deficient mouse brain cortical synaptosomes $[Ca^{2+}]_i$ increase in response to K^+ (30 mM) depolarization was 42% higher than in wild-type. ω-agatoxin IVA 0.2 μM decreased Ca^{2+}-influx in neocortical wild-type synaptosomes by 53%, and abolished differences between gsn+/+ and −/− genotype. K^+-induced release of glutamate in neocortical synaptosomes was 78% higher and lesion size after MCA occlusion was 45% higher than in wild-type.

It is concluded that presynaptic Ca^{2+} influx is increased in gsn deficient nerve terminals which, together with subsequently increased glutamate release, increases neuronal vulnerability.

Perfusion and molecular diffusion MR-imaging of the brain: *In vivo* assessment of tissue alteration in cerebrovascular and neurodegenerative diseases

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The combined used of perfusion imaging (PI) and molecular diffusion imaging (DWI) are opening a new window into the processes that occur during the first hours of ischemia. DWI detects early changes of proton diffusion associated with cytotoxic edema. PI has the potential to characterize the degree of regional hypoperfusion. Mismatches between DWI and PI, i.e. hypoperfused areas with normal ADC are considered potentially salvageable. We present results of 11 patients with an angiographically defined thrombembolus in the middle cerebral artery and a spontaneous stroke evolution. Whereas the infarct core was clearly visible on both DWI and PI, tissue at risk of infarction could only be detected by an increased blood volume and transit time. However only in a subgroup of patients ($n = 3$) these areas were incorporated into the final infarct. In these patients perfusion parameter of tissue at risk of infarction were more pronouncedly altered than in those where the tissue at risk was spared from infarction (ratios of tissue at risk vs normal (rCBV 2.17 ± 0.59, MTT 1.67 ± 0.22, TTP 1.32 ± 0.11, $p < .05$). These human data show that a detailed analysis of diffusion/perfusion mismatches allow the identification of tissue at risk of damage.

Inhibition of phosphatidylcholine synthesis and excitotoxic cell death in cerebellar granule cell cultures

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Glucose deprivation enhances the sensitivity of cerebellar granule cells to die by excitotoxicity. We have previously reported that neither 70 min of glucose deprivation, a treatment that depletes cell energy resources, nor exposure to 20 μM glutamate (GLU) for 30 min, induce significant cell death in cerebellar granule cell cultures, 24 h after treatment. In contrast, the combined treatment with 20 μM GLU and glucose deprivation induces both cell death and choline (Cho) release. We investigated whether the neurotoxic effect of this treatment is related with inhibition of phosphatidylcholine (PC) synthesis. We found that exposure to 20 μM GLU alone for 30 min, glucose deprivation for 70 min, and the combination of both

treatments inhibited PC synthesis when measured at the end of treatment by 71%, 92% and 91%, respectively. Furthermore, we found that exposure to either 20 μM GLU, glucose deprivation or 20 μM GLU + glucose deprivation decreased incorporation of [^{3}H]Cho into phosphocholine and increased the intracellular content of free [^{3}H]Cho, indicating that all these treatments inhibit the synthesis of PC by inhibiting choline kinase activity. Since only the combined treatment with 20 μM GLU plus glucose deprivation evoked Cho release and excitotoxic cell death, the present results indicate that other factors in addition to inhibition of PC synthesis are required to induce Cho release and excitotoxic cell death in cerebellar granule cells.

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The role of striatal metabotropic glutamate receptors in degeneration of dopamine neurons

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The present study was undertaken to characterize the effect of blockade of the $mGlu_5$ receptor subtype by 2-methyl-6-phenylethynylpyridine (MPEP), as well as the effect of stimulation of the $mGlu_{2/3}$ receptor by (−)-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylic acid (LY379268) on spontaneous and stimulated dopamine (DA) release in rat striatum using an *in vivo* microdialysis. MPEP (100–500 μM), perfused through a microdialysis probe affected neither the basal nor the veratridine (100 μM)-stimulated striatal DA release. However, MPEP given intraperitoneally (5 mg/kg) diminished either the basal or the veratridine-evoked DA release. LY379268 (100–250 μM) administered locally also inhibited the veratridine-evoked DA release in rat striatum.

Antagonists of mGluR-I and agonists of mGluR-II have been shown to have neuroprotective properties in several models of neurotoxicity in animals. We have approached this issue using a selective $mGlu_5$ antagonist in an animal model of neurotoxicity induced by methamphetamine. In our preliminary experiments, methamphetamine (5 × 10 mg/kg *sc* every two hours) decreased the tissue content of striatal DA and its metabolites DOPAC and HVA.MPEP (5 × 5 mg/kg ip) given before every methamphetamine injection reversed its action.

The effect exerted by the $mGlu_5$ antagonist MPEP seem to be mediated by sites located outside the striatum due to relieving DA neurons of the facilitatory influence of glutamate. In turn, the attenuation of DA release from nigrostriatal terminals by LY379269 may be a consequence of activation of striatal $mGlu_{2/3}$ receptors. Reversal of the methampetamine-induced DA depletion suggests a potential for neuroprotective activity of MPEP.

Molecular imaging of perfusion disturbances in normal-tension glaucoma

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Glaucoma remains a major eye illness with unknown etiology. Although elevated intraocular pressure has been shown to be the major risk factor, there is a cohort of relatively young patients developing normal-tension glaucoma (NTG). Assymptomatic ischemic events in brain have been shown to be often attributable to galucoma. Perfusion of the retina and optic nerve head suffering from observed vasospastic dysfunction may be further reduced by changes in the intraocular pressure. Ocular ischemia developed due to these blood flow deficits may play a major role in initiation of glaucoma. Possibly secondary to ischemia the autoimmunogenic capacity is activated by NTG patients having an increased prevalence of systemic autoimmune diseases. Therefore, the determination of potential molecular markers in blood lymphocytes could be useful for early diagnostics of NTG.

Our recent study using "gent hunting"-techniques showed indeed altered gene expression in lymphocytes of NTG patients. The demonstrated downregulation of XPGC gene expression which subsequently leads to the accumulation of damaged DNA and an elevated p53 expression, together with the upregulation of a new ABC-transporter seem to be specific for the pathogenesis of NTG. Molecular imaging of NTG provides insights in mechanisms of disease initiation and allows the early diagnostics and preventive treatment.

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Glutamine/glutamate metabolism and neurotoxicity under hypoglycemic conditions in vitro

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Aggregate cell cultures prepared from fetal rat telencephalon were used to study neuronal amino acid consumption during glucose restriction. To that end, both mixed (neuron-glia) and neuron-enriched cultures were grown in chemically defined medium and tested at an advanced maturational stage. It was found that 6 h of exposure to reduced glucose (0.25 mM instead of 25 mM) caused significant increases in the consumption of several amino acids and the accumulation of ammonia. It also greatly changed the intracellular level of several amino acids in neurons, particularly of aspartate and glutamate. Irreversible neuron-specific damage was observed one week after the insult. Elevated glutamine media concentrations (4 mM instead of 0.25 mM) during glucose restriction further increased ammonia production and neuronal damage, although the overall rate of glutamine metabolism remained practically unchanged. Taken together, our findings suggest that glucose deficiency caused (i) the dysfunction of crucial transamination pathways; (ii) a shift towards the oxidative deamination of glutamine and several other amino acids used by neurons as alternative energy substrates; and (iii) the accumulation of neurotoxic ammonia levels.

L-Dopa – The success story of a "biologically inactive" amino acid

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The racemic (D,L) mixture of the naturally occurring neutral aromatic amino acid 3,4-dihydroxy-L-phenylalanine (L-dopa) was first synthetized in 1911. In 1913, the natural levorotatory isomer was isolated from Vicia faba beans and declared to be biologically inactive. However, in 1930 L-dopa was observed to lower the blood pressure in the rabbit, an effect opposite to the vasopressor effect of adrenaline. Following the discovery, in 1938, of the enzyme L-dopa decarboxylase, L-dopa's conversion in tissues (by decarboxylation) to dopamine (DA), the first biologically active substance in the biosynthetic pathway of catecholamines, was demonstrated. Subsequent

pharmacological studies, done between 1942 and 1957, showed that the biological actions of L-dopa were, in principle, due to DA formed from it in the body. In 1957, the central antireserpine effect of D,L-dopa was described in mice and confirmed in 1960 with L-dopa in humans. Following the demonstration of DA's occurrence in the brain in the years 1957/58, D,L-dopa was found (in rabbits) to restore brain DA levels, reduced by reserpine. In 1960, the severe brain DA deficit, confined to patients with Parkinson's disease (PD) was reported and a year later L-dopa's superior anti-akinesia effect in patients with PD demonstrated. Finally, in 1967 the high-dose oral L-dopa regimen was successfully introduced into clinical practise. In contrast to these supreme achievements, two related early studies remained, for different reasons, without consequence. Despite some initial doubts about its mechanism of action, there is now convincing evidence for L-dopa therapy being a classic example of a central neurotransmitter replacement therapy, with the severe brain DA deficit furnishing a rational basis for the amino acid's clinical use and high efficacy in patients suffering from PD.

Domoic acid neurotoxicity in hippocampal slice cultures

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The toxicity of domoic acid (DOM) was studied in rat hippocampal slice cultures, prepared from 7-days old rats and grown on semipermeable membranes for 2–3 weeks before exposure. DOM (0.1–100 μM) was added to the medium, alone or together with the glutamate receptor antagonists NS-102, NBQX or MK-801, for 48 hrs followed by 48 hrs in normal medium. Neuronal degeneration was monitored and EC_{50} values estimated by densitometric measurements of the cellular uptake of the propidium iodide (PI) at 24, 48, 72 and 96 hrs.

The lowest EC_{50} values, obtained at 72 hrs, were: CA1 (6 μM), dentate granule cells (DG) and CA3ab (10 μM),CA3c (12 μM). Protective effects of 10 μM NBQX at 72 hrs were seen against 3 μM DOM in DG, CA1 and CA3c and against 10 μM DOM in CA1 and CA3c. 10 μM NS102 and MK801 only displayed protective effects together with NBQX. MK801 thus significantly increased the protective effect of 10 μM NBQX in CA1 against 10 μM DOM in combination with 10 μM NBQX and 10 μM NS102. We can confirm that DOM neurotoxicity primarily involves AMPA/kainate receptors, but also NMDA receptors at high concentrations (glutamate release).

Glutamatergic mechanisms in addiction

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Although dopamine has been most clearly tied to the development of addiction to drugs of abuse, recent studies indicate that once addiction has been established the expression of addictive behaviors, such as drug craving, is mediated more by long-term neuroadaptaions in glutamate transmission. Data will be presented which supports and extends this hypothesis. Repeated cocaine injections were given for one week and three weeks after the last drug injection a number of molecular, neurochemical and behavioral neuroadaptations were measured. It was found that in the nucleus accumbens there is a increase in the expression of genes encoding mGluR2/3 and GluR1, and a decrease in the expression of mGluR5 and its accompanying scaffolding proteins Homer1bc. This was accompanied by an increase in the capacity of mGluR2/3 receptors to regulate presynaptic glutamate release and a blunting in the effects of stimulating mGluR1/5 receptors. In addition, there is reduced activity in the cystine/glutamate exchanger 3 wks after repeated cocaine. As a result of these changes there is a decrease in the basal release of glutamate, and a relative increase in the releasibility of glutamate upon stimulation. By using the reinstatement model of drug seeking behavior, it was shown that glutamate transmission in the projection from the prelimbic cortex to the core of the nucleus accumbens was particularly affected by the cocaine-induced changes in gene expression. Taken together, these findings support the use of glutamate autoreceptor agonists as possible therapeutic adjuvants in treating the cravings associated with addiction.

Suppression of spontaneous hydroxyl radical formation in dopamine-denervated neostriatum, but enhancement by glutamate

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Dopamine (DA), a catechol that autoxidizes to an *o*-quinone, is implicated as an endogenous pro-toxin, However, the following studies suggest that DA has dual neurodegenerative and neuroprotective roles. In rats treated as neonates with 6-hydroxydopamine (6-OHDA; 134 :g icv), there was a 99% reduction in striatal tissue DA content in adulthood, and a 3 to 5-fold increase in spontaneous hydroxyl radical (HO*) formation (indirect salicylate trapping method: dihydroxybenzoic acid analysis). Additionally, systemic L-DOPA (60 Mg/kg i.p.) suppressed HO* formation. However, when glutamate (50 mM) was added to an in vivo microdialysate, HO* formation was increased substantially more in the microdialysate from DA-innervated striatum. These findings indicate that DA innervation is inherently neuroprotective, but in the presence of a high level of an excitatory amino acid, DA innervation predisposes to formation of reactive oxygen species. Ongoing neuronal activity is likely to interact with and to determine the role of DA as a neurotoxic or neuroprotective substance.

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Glutamatergic mechanisms in schizophrenia

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The glutamate hypothesis of schizophrenia along with the dopamine hypothesis was intensively discussed in the past. The last years however suggest more and more that neither a hypofunction of the glutamatergic system alone nor a hypofunction of the dopaminergic system alone is responsible for symptoms found in schizophrenia.

The basal ganglia (BG) as the critical structures mediating symptoms of schizophrenia are innervated by dopaminergic fibers from the mesencephalon as well as by glutamatergic fibers from limbic structures; like prefrontal cortex, hippocampus, entorhinal cortex and amygdala. Thus, limbic input is able to modulate information processing in each structure of the BG and by this way control dopaminergic functions through feedback mechanisms. Dysfunction in limbic structrues may result

in an imbalance of information processing via the BG and terminates in behavioral symptoms of schizophrenia.

We showed in recent neurochemical studies in combination with behavioral analysis that a simple, generalized hypofunction of limbic glutamatergic input on BG nuclei is not the key mechanism inducing schizophrenic behavior. A dysfunction of a particular limbic structure or pathway seems to be responsible for an imbalanced information processing via the BG and imbalanced behavioral adaptation terminating in schizophrenic symptoms.

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Characterization of allosteric modulators of the metabotropic glutamate receptor subtype 5

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There is a need to identify subtype-specific ligands for mGlu receptors to elucidate the potential of these receptors for the treatment of nervous system disorders. To date, most mGluR antagonists are amino acid-like compounds acting as competitive antagonists at the glutamate binding site located in the large extracellular N-terminal domain.

We have investigated novel subtype-selective mGluR5 antagonists which are structurally unrelated to competitive mGluR ligands. Using a series of chimeric receptors and point mutations we demonstrate that these antagonists interact with novel allosteric binding sites in the TM domain via a noncompetitive mechanism of action. Recent studies in animal models implicate $mglu_5$ receptors as a potentially important therapeutic target particularly for the treatment of pain and anxiety.

Role of VEGF in an experimental model of cortical micronecrosis

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Vascular endothelial growth factor (VEGF) is a major mediator in angiogenesis and vascular permeability. In central nervous system (CNS) VEGF plays pivotal roles such e.g., inductor of endothelial cell proliferation, migration and inhibition of apoptosis, as well as mediator of blood brain barrier (BBB) breakdown and subsequently of brain edema formation. These ubiquitous epiphenomena are major complications in several CNS pathologies, including head trauma and stroke.

Reduced tissue oxygen tension (hypoxia) and hypoglycaemia triggers VEGF expression that occurs in ischemic regions around postraumatic or postinfarct necrosis.

After brain injury, the expression of VEGF is increased contributing to disruption of the BBB. VEGF increases the permeability of BBB via the synthesis/release of nitric oxide and subsequent activation of soluble guanylate cyclase. The immunohistochemistry shows an increase of stained astrocytes around a cortical micronecrosis. VEGF participates in the response of the CNS to injury in a dose dependent way. Immunostaining correlates with infarct volume and clinical disability. VEGF-antagonists reduce ischemic brain edema and injury, involving VEGF in pathogenesis and eventually in treatment of stroke and related disorders. This cytokine also exerts a neuroprotective effect mediated by its receptor flk-1. Functions related to the inflammatory response, co-expression with proteins of the ECM and interaction with the two main receptors, flk-1 and flt-1, will be discussed.

Intrinsic survival pathways in neurons: Signaling mechanisms and the role of N-methyl-D-aspartate receptor variants

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N-Methyl-D-aspartate (NMDA) receptors can mediate excitotoxic or neuroprotective responses. One of the molecular mechanisms responsible for NMDA neuroprotection involves the release of brain-derived neurotrophic factor (BDNF) which in turn binds to and activates its cognate receptor TrkB. BDNF levels in the neuronal culture medium increased 2-fold when cells were preincubated for three hours with NMDA. At three hours, the increase in BDNF protein levels in the medium was accompanied by a concomitant increase in BDNF mRNA. Thus, NMDA elicited two temporally distinct responses: an early release of BDNF protein followed by a later transcriptional activation of DBNF mRNA and protein release. These results suggest that NMDA activates the TrkB receptor via a BDNF autocrine loop resulting in neuronal survival. In addition, extracellular regulated kinases (Erk 1/2) were rapidly activated, which peaked within six hours of NMDA treatment. Erk 1/2 activation is completely blocked by MK-801 and partially blocked by K252a, suggesting the NMDA and TrkB receptors act in a coordinated fashion to activate Erk 1/2. As an extension of this work, we discovered a single nucleotide polymorphism in the human NR1 gene that, when transfected into HEK cells, alters the electrophysiological properties of the NMDA receptor complex. Possible consequences of this NMDA receptor variant in signaling will be discussed.

Submolecular adventures of brain tyrosine: what we are searching for now?

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This overview summarizes our recent knowledge of the role that tyrosyl radical ($TyrO^{\bullet}$) can play in neurochemical systems of brain and thereby lead to neural disorders (PD, AD, ALS). These could involve the interactions of tyrosine and $TyrO^{\bullet}$ with reactive oxygen species (ROS) and reactive nitrogen species (RNS), via radical mechanisms and chain processes in the presence of O_2 and endogenous brain antioxidants. Concentrations of $TyrO^{\bullet}$, ROS and RNS can increase dramatically under conditions of generalized stress: oxidative, nitrative or reductive. This in turn can directly damage (by lipid peroxidation) or indirectly damage (by protein oxidation and/or nitration) cellular substructures which ultimately can lead to apoptotic neuronal cell death or autoschizis. Enzymatically (classical peroxidase mechanisms) or non-enzymatically formed $TyrO^{\bullet}$ can react with $NO^{\bullet}$ and this reversible and intrinsic "combination" acts to "buffer' $TyrO^{\bullet}$ concentrations. The reaction of $TyrO^{\bullet}$ with superoxide ($O_2^{\bullet -}$) is a scavenging reaction which proceeds rather by addition, not by electron transfer; and major resultant products are tyrosine hydroperoxides (TyrOOH). However, the decay of $TyrO^{\bullet}$ can be also terminated by self-termination (dimerization) resulting in dityrosine (DT) formation. $TyrO^{\bullet}$ can catalyze LDL oxidation, although the precise mechanisms of this reaction *in vivo* remain unknown. Nitration of tyrosine to 3-nitrotyrosine (3-NT) requires a one-electron oxidation as a primary step, with formation of $TyrO^{\bullet}$, followed by addition of the nitrogen dioxide radical ($NO_2^{\bullet}$). The promoting effect of carbon dioxide on peroxynitrite-mediated tyrosine nitration

(via radical mechanisms) ($TyrO^{\bullet}/NO^{\bullet}/O_2^{\bullet-}/NO_2^{\bullet}$ system) is due to the selective reactivity of the putative carbonate radical anion, as compared to that of the oxidizing hydroxyl radicals ($^{\bullet}OH$). Moreover, once formed, 3-NT may act to promote repetitive redox cycling; it may be reduced to the corresponding nitroanion radical, which is then oxidized by molecular O_2 to $O_2^{\bullet-}$ and parent 3-NT. One-electron oxidation of 3-NT can result in catalytically active imminoxyl radical.

DT formation can outcompete tyrosine nitration at low-steady state concentrations of peroxynitrite. It is unquestionable that very high fluxes of $NO^{\bullet}$ and $O_2^{\bullet-}$ are requisite intermediates of peroxynitrite, a tyrosine nitration agent formed via $TyrO^{\bullet}$. Evidence for the existence of generalized stress within neurons includes the presence of protein peroxides (TyrOOH), DT, and 3-NT. The nitration/denitration processes can be pathologic, but these also may play: 1) a signal transduction role; 2) a role of "blocker" for radical-radical reactions (scavenging of $NO^{\bullet}$, $NO_2^{\bullet}$ and $CO_3^{\bullet-}$ by $TyrO^{\bullet}$); or 3) a role of delimiting factors for peroxynitrite formation.

It is still unknown whether oxidation/nitration of tyrosine (as dopamine precursor or protein residue) via $TyrO^{\bullet}$ formation, is a footprint of generalized stress and neuronal disorders, an important part of $O_2^{\bullet-}$ and $NO^{\bullet}$ metabolism, or just a part of integral processes for maintaining neuronal homeostasis. The complete answer of these questions should be the first priority task of our recent search, wherein the problem of increased free radical formation in the brain and/or the imbalance of ratios: ROS/RNS/$TyrO^{\bullet}$ may be all important in determining neural cell and tissue injuries under pathological conditions resulted from generalized stress.

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Molecular imaging of early events in the development of the diabetic cardiomyopathy

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More than 50% of patients with type 2 diabetes have coronary heart disease, related to silent ischemia, caused by an autonomic denervation of the heart in diabetic patients. Oxidative damage to DNA has been well documented in cardiac cells isolated from diabetic patients and rats with streptozotocin-induced *Diabetes mellitus* (DM). This DM-model shows already seven days after onset of disease structural changes in vascular tissue typical for the development of atherosclerosis. This study evaluates possible molecular mechanisms for early events in the development of DM-induced cardiomyopathy.

Methods: Using "Expression Array" we examined the activation of cardiac cell death in heart of DM-rats. MS-PCR was used to examine a differential DNA methylation.

Results: An increased expression of genes encoding renin, angiotensinogen and p53 was detected in heart of DM-rats. Substantial changes in the methylation status of the p53-dependent $p21^{WAF1/CIP1}$-gene and the cyclin D1-gene were detected in DM-rats.

Conclusions: The renin-angiotensin system is upregulated with diabetes, and this may contribute to the development of cardiomyopathy via oxidative damage and p53-dependent activation of cardiac cell death. This pathway includes *de novo* methylation of the p53-inducible $p21^{WAF1/CIP1}$-gene encoding a protein which binds to and inhibits a broad range of cyclin-cyclin-dependent kinase complexes.

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Growth hormone and neuroprotection

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During the past decade studies have indicated that growth hormone (GH) may exert effects on the central nervous system (CNS). For instance, GH replacement therapy was found to improve the psychological capabilities in adult GH deficient (GHD) patients. Furthermore, beneficial effects of the hormone on certain functions, including memory, mental alertness, motivation and working capacity have been reported. Likewise GH treatment of GHD children has been observed to produce significant improvement in many behavioural problems seen in these individuals. Studies also indicated that GH therapy affects the cerebrospinal fluid (CSF) levels of various hormones and neurotransmitters. Further support that the CNS is a target for GH emerges from observations indicating that the hormone may cross the blood-brain-barrier (BBB) and from studies confirming the presence of GH receptors in the brain. It was previously shown that specific binding sites for GH are present in discrete areas in the CNS of both humans and rats. In peripheral tissues GH is shown to elicit its effects through a second mediator insulin-like growth factor 1 (IGF-1). IGF-1 is well recognized as a protective agent against neural injury in the CNS. The neuroprotective effect of this peptide has a broad spectrum affecting many brain regions and acts through its anti-apoptopic effect. The production of IGF-1 is upregulated in areas of brain damage and the IGF-1 system may be an important part of an endogenous neuroprotective system. In spinal cord injuries, however, the content of IGF-1 is reduced. We recently observed a neuroprotective effect of topical application of IGF-1 in animals subjected to spinal cord trauma. The observed effect may be mediated via a mechanism involving nitric oxide. In the same animal model we have very recently observed a neuroprotective effect of GH. Recent reports suggest that the level of GH is drastically reduced in patients with spinal cord injury. In victims of spinal cord injury the secretion of GH and IGF-1, as well, is known to be decreased. Therefore, exogenous substitution of GH and IGF-1 might be a promising approach in the future therapy of spinal cord injury victims. In fact, there is one report indicating that prolonged treatment with synthetic GH of spinal cord injured rats attenuates some of the neurological motor dysfunction seen in these animals 3 weeks following trauma. In our animal model we observed that topical application of rGH significantly reduced trauma-induced disturbances in the fluid micro-environment. We also noted that GH was capable of attenuating the trauma-induced depression of spinal cord evoked potentials. The mechanism by which GH exerts it neuroprotective effects will be discussed.

Glutamate-mediated striatal dysregulation and pathogenesis of Parkinsonian motor response complications

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Chronically administered levodopa in Parkinson's disease (PD) treatment is ultimately associated with alterations in motor response. In 6-hydroxydopamine lesioned hemiparkinsonian rats, chronic twice-daily administration of

levodopa progressively shortens duration of contralateral turning and augments the period of turning at or below 20% of peak turning rate. The pathogenesis of the response alterations involves in part sensitization of the corticostriatal glutamatergic synaptic activity. Characteristic changes involving interactions between striatal kinase and phosphatase signaling now appear to contribute to sensitization of spiny-neuron glutamatergic receptors. Glutamate-mediated striatal dysregulation, subsequently, modifies basal ganglia output system in ways that favor the appearance of Parkinsonian motor response complications. At a molecular level, transcriptional activation of striatal CREB contributes to the persistent expression of the levodopa-induced motor response alterations. Conceivably, a safer and more effective therapy for all stages of PD can be provided by drugs that target intracellularly on striatal kinases or phosphotases, or by agents that interact extracellularly on non-dopaminergic striatal receptors such as AMPA and NMDA, adenosine A2, adrenergic a2, opiod, and serotonergic 2B.

The role of striatal metabotropic receptors in Parkinson's disease

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The primary cause of Parkinson's disease is a loss of dopamine in the corpus striatum. It has been postulated that this effect leads to disinhibition of the striopallidal pathway and, secondarily, to a functional shift towards glutamatergic stimulation. The aim of the present study was to find out whether inhibition of glutamatergic transmission at a level of metabotropic glutamate receptors (mGluRs) in the striatum may alleviate parkinsonian-like symptoms in rats.

The non-competitive antagonist of receptor subtype 5 (mGluR5), MPEP (1.0–10 mg/kg ip), or the agonist of group II mGluRs, LY354740 (5–10 mg/kg ip), reduced the haloperidol-induced muscle rigidity and catalepsy. Intrastriatal injections of the antagonist of mGluR1, (RS) AIDA (7.5–15 μg/0.5 μl), but not of the agonist of group II mGluRs, 2R,4R-APDC (7.5–15 μg/0.5 μl), inhibited the muscle rigidity induced by haloperidol. In order to search for an influence of mGluRs on the striopallidal pathway, the effect of MPEP or of the agonist of group II mGluRs, DCG-IV, on the preproenkephalin mRNA expression in the striatum was examined.

The obtained results suggest that blockade of group I mGluRs, or stimulation of group II mGluRs may be important to the amelioration of parkinsonian symptoms. Striatal mGluRs may contribute to at least some of these effects.

On the role of group I metabotropic glutamate receptors (mGluR) in the mechanism of action of antidepressant drugs

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Several lines of evidence suggest an important role of glutamate in depression. The involvement of group I mGluRs in depression has also been proposed. Thus, we decided to evaluate whether group I mGluRs antagonists have antidepressant-like effects. We also investigated if antidepressant treatment influences group I mGlu receptors in the brain.

The experiments were performed on male Wistar rats (200–250 g) and male C57BL/6 mice (22–26 g). AIDA (group I mGluRs antagonist) given i.v. in the dose of 50 μg, decreased the immobility time in the despair test in rats. MPEP (noncompetitive, systemically active mGluR5 antagonist) given i.p., was not effective in the despair test in rats. However, in doses of 1.0, 10 and 20 mg/kg, it significantly decreased the immobility time of mice in the tail suspension test. Moreover, the deficit in passive-avoidance learning, which was observed in bulbectomized rats, was reversed by chronic, but not acute MPEP (10 mg/kg) treatment. Prolonged imipramine treatment resulted in significant increase of the level of expression of mGlu5 receptors in the CA1 field of the hippocampus, while prolonged electroconvulsive shock treatment (ECT) enhanced significantly the chemiluminescence of mGlu5 receptors in the CA3 field. The results indicate that group I mGlu receptors are modified by chronic antidepressant treatment and that group I metabotropic glutamate receptors antagonists may play a role in the therapy of depression.

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Glutamatergic mechanisms in dependence and withdrawal

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Chronic exposure to nicotine, alcohol, opioids, sedatives, and cannabis results in development of drug dependence that becomes evident upon a cessation of drug administration and expresses itself as a withdrawal syndrome (with its physiological and motivational manifestations). Adaptations at the N-methyl-D-aspartate receptor (NMDA-R) complex have been observed in different brain areas during chronic exposure to, and upon withdrawal from, opioids, ethanol, benzodiazepines and barbiturates. Behavioral studies employ the assessment of the effects of NMDA-R antagonists on: a) the development of dependence (NMDA-R antagonists are co-administered with the drug), b) the maintenance of dependence (NMDA-R antagonists are administered to animals with pre-established dependence, and – most relevant to the clinical situation – c) on the expression of drug dependence (assessment of the withdrawal severity in subjects with NMDA-R antagonists administered just before the expected emergence of withdrawal). The development of dependence to opioids and benzodiazepines is significantly retarded by NMDA-R antagonists. Studies from this laboratory demonstrate similar inhibition by NMDA-R antagonists of the maintenance of opioid dependence. Both in rodents and humans, the expression of opioid antagonist-precipitated as well as spontaneous (natural) withdrawal is inhibited by NMDA-R antagonists, and animal data demonstrate similar inhibition of the expression of dependence produced by ethanol, barbiturates and benzodiazepines.

Antioxidant compound H-290/51 modulates glutamate and GABA immunoreactivity in the rat spinal cord following trauma

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The involvement of the excitatory amino acid, glutamate and the inhibitiory amino acid, gamma-amino butyric acid (GABA) in the pathophysiology of spinal cord trauma is not known in details. This investigation is focused on the involve-

ment of glutamate and GABA in a rat model of spinal cord injury using immunohistochemistry. Spinal cord injury induced by an incision into the right dorsal horn of the T10–11 segments resulted in profound edema formation and cell damage in the adjacent T9 and T12 segments at 5 h. Pretreatment with H-290/51 (50 mg/kg, p.o.), a potent antioxidant compound, effectively reduced the edema formation and cell injury following trauma. At this time period, untreated traumatised rats exhibited a marked increase in glutamate immunoreactivity and a distinct decrease in GABA immunostaining in the T9 and T12 segments compared to the control group. The changes in glutamate and GABA immunoreactivity in traumatised rats were considerably attenuated by pretreatment with H-290/51. These results suggest that (i) oxidative stress contributes to alterations in glutamate and GABA in spinal cord injury, (ii) glutamate and GABA are contributing to edema formation and cell damage and (iii) the antioxidant compound H-290/51 has a potential therapeutic value in the treatment of spinal cord injuries.

Glutamatergic mechanisms in depression

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Both preclinical (i.e., behavioral despair models) and clinical studies indicate that compounds reducing transmission at NMDA receptors are antidepressant. Conventional antidepressants may be viewed as "monoamine-based", increasing the synaptic availability of serotonin, norepinephrine, and/or dopamine. However, chronic administration of of conventional antidepressants alters both mRNA levels encoding NMDA receptor subunits and radioligand binding to this family of ligand-gated ion channels in circumscribed areas of the CNS indicating that NMDA receptors may be a downstream target of these monoamine-based agents. We have recently reported (Li, et al., Neuropharmacology, in press) that a class of AMPA receptor potentiators also exhibits antidepressant-like actions in preclinical models. In this presentation, I will describe how these two distinct, and (at a cellular level) seemingly diametric approaches employing glutamatergic mechanisms converge on intracellular targets that are also impacted by chronic treatment with biogenic amine-based agents.

Comparison of the *in vitro* and *in vivo* neurotoxicity of three new sources of kainic acid

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Kainic acid is an essential pharmacological tool for many forms of neurobiological research. Until several years ago, all commercially available kainic acid was derived from a single biological source (*Digenia simplex*). Commercial isolation of kainic acid in Japan ceased in 1999, creating a void in the marketplace. Recently several different companies have become providers of kainic acid, but each uses a different source of the compound (2 biological and 1 synthetic) and different isolation procedures. Our objective was to use three common assay systems to evaluate the comparative pharmacological and neurotoxicological properties of these three sources of kainic acid. Dose response curves, both alone and in the presence of receptor selective antagonists, were constructed for each kainate formulation using (a) cerebellar granule neurons in culture, (b) isolated hippocampal slice preparations, and (c) whole animal behavioural toxicity studies. Preliminary results reveal many similarities, but also distinct differences between the three formulations, especially when challenged with antagonists for different EAA receptors. Full results will be presented and discussed with respect to their implications for both extending the known kainite literature and for future studies employing kainic acid as a ligand in both mechanistic investigations and in animal models of neurodegenerative disease.

Expression of cell-cycle related proteins and excitotoxic neurodegeneration

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Induction of cell-cycle proteins has been found in several *in vivo* models of excitotoxic neurodegeneration, including focal or global ischemia and kainate treatment (see Copani et al., TiNS 24:25–31, 2001 as review).

Our results from *in vitro* studies further elucidate the role of cell-cycle related proteins in neuronal apoptosis induced by excitotoxins. Exposure of primary cerebellar neurons to toxic concentrations of glutamate was found to produce a significant, short lasting increase in the expression of p53 and cdc2. Transcriptional activity of p53 was shown by increased p53 DNA binding activity and by the concomitant induction of the cdk inhibitor p21, the cell cycle regulator GADD 45 and the apoptotic induced Bax.

Cell-cycle proteins are also expressed concomitantly to DNA damage in neurons undergoing excitotoxic degeneration. We found that excessive activation of glutamate receptor by NMDA results in the formation of 8-OH-deoxyguanosine, which is a marker of oxidative DNA damage. In addition, the expression of the DNA repair factor MSH2 increases in cultured cerebellar neurons or in CA3 pyramidal cells that have been challenged with excitotoxins.

Excitotoxicity may thus provide a further example of how re-expression of cell-cycle proteins might be tightly connected to DNA damage and repair in neurons.

Levodopa-associated dyskinesias: clinical features and treatment strategies

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Patients with Parkinson's disease by definition benefit from levodopa therapy. However, after 5 years of therapy 50% of patients experience motor response complications (MRC's): the benefit from each dose becomes shorter (wearing-off), more unpredictable (on-off) and associated with involuntary movements (dyskinesias). When dyskinesias first arise, they are associated with high levodopa levels and may be prevented or minimized by lowering levodopa intake. Later on, the therapeutic window of levodopa narrows progressively and dyskinesias occur at doses equal to those needed to induce an antiparkinson effect. While the pathogenesis of motor complications remains incompletely understood, recent clinical studies implicate mechanisms downstream from the degenerating nigrostriatal dopamine system, possibly involving glutamatergic

projections to the basal ganglia. In a rat model of PD, blockade of striatal glutamate receptors of the N-methyl-D-aspartate (NMDA) subtype reverses levodopa-induced motor fluctuations. Similarly, in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) lesioned primates, several NMDA-antagonists reduce levodopa-associated dyskinesias. In parkinsonian patients the NMDA-antagonists dextromethorphan, dextrorphan and amantadine improve dyskinesias as well. These findings have lead to the suggestion that hyperfunction of NMDA receptors on striatal efferent neurons, as a consequence of chronic non-physiologic dopaminergic stimulation, contributes to the pathogenesis of motor response complications.

Modeling Alzheimer's disease and other proteopathies

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Protein misfolding and aberrant polymerization are salient features of virtually all central neurodegenerative disorders, including Alzheimer's disease (AD), Parkinson's disease, polyglutamine diseases, tauopathies, and prion diseases. In many instances, a single amino acid change can predispose to disease by increasing the production and/or changing the biophysical properties of a specific protein. Possible pathogenic similarities among the cerebral proteopathies suggest that therapeutic agents interfering with the proteopathic cascade might be effective against a wide spectrum of diseases. However, testing compounds preclinically will require disease-relevant animal models. Numerous transgenic mouse models of AD-like pathology have now been produced. Our studies have found that Tg2576 mice overexpressing human β-amyloid precursor protein (HuAPP695K670N/M671L) produce copious deposits of diffuse and compact β-amyloid as they age, and that females are more susceptible than are males (Callahan et al., Am. J. Pathol. 158, 1173–1177, 2001). Recently, we also found that the overexpression of p25 protein, an activator of the kinase cdk5, results in tau hyperphosphorylation, axonopathy and severe motor deficits in transgenic mice, in the absence of neurofibrillary tangles. None of the existing transgenic models of β-amyloidosis or tauopathy fully recapitulates the pathology of AD. In an attempt to more authentically model the human disease, we infused dilute AD-brain extracts into TG2576 mice at 3-months of age (i.e. 5–6 months prior to the usual onset of β-amyloid deposition). We found that infusion of AD brain extracts results in: 1) Earlier and more abundant deposition of β-amyloid in βAPP-transgenic mice (Kane et al., J. Neurosci. 20, 3606–3611); 2) evidence for the spread of pathology to other brain areas, possibly by neuronal transport mechanisms; and 3) tau hyperphosphorylation (but not neurofibrillary pathology) in axons passing through the injection site. The seeding of β-amyloid by AD brain extracts suggests pathogenic similarities between β-amyloidoses such as AD and other cerebral proteopathies, and could provide a new model for studying the proteopathic cascade and its neuronal consequences in neurodegenerative diseases. Supported by Warner-Lambert/Pfizer.

Effects of dietary deficiency of selective amino acids on the function of the cornea and eye lens in rats

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Purpose: The effects of essential amino acid deficiencies on function of cornea and lens were investigated.

Methods: Dietary deficiencies of tryptophane and methionine were studied in young rats over 3 months. Transparency of cornea and lens were evaluated using slitlamp microscope and Scheimpflug camera. After sacrifice, lens fresh weight and crystallin patterns were determined to evaluate effects on lens growth and protein synthesis.

Results: Methionine deficiency had no effect on the parameters investigated. Tryptophane deficiency caused severe loss of body weight in both rat strains (Brown-Norway, BN; Sprague-Dawley, SD), SD rats also lost their hair. They developed corneal neovascularisations and cortical cataracts. BN rats developed faint neovascularisations and a discontinuity zone in the lens. Diet intermission arrested pathological processes restarting when feeding diet again. This observation is supported by lens fresh weight data. DNA staining evidenced that tryptophane deficiency arrested lens fiber maturation.

Conclusion: A difference has been found for 2 essential amino acids in their effects on transparency of cornea and lens. Tryptophane deficiency stimulated corneal neovascularisation, but arrested lens fiber cell maturation. The difference in reaction of cornea and lens to tryptophane deficiency between BN and SD rat eyes remains to be elucidated.

Neuroprotective effects of dynorphin antiserum in spinal cord injury

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Dynorphin is a neuropeptide that is present in the dorsal horn of the spinal cord. The peptide is actively involved in pain processing pathways. However, its involvement in spinal cord injury is not well known. Alteration in dynorphin immunoreactivity occurs following a focal trauma to the rat spinal cord. Infusion of dynorphin into the intrathecal space of the cord results in ischemia, cell damage and abnormal motor function. Antibodies to dynorphin when injected into the intrathecal space of the spinal cord following trauma improves motor recovery and reduces edema and cell changes. However, influence of dynorphin on trauma induced alteration in spinal cord bioelectrical activity is still not known. Spinal cord evoked potentials (SCEP) are good indicator of spinal cord conduction that are altered following trauma. Therefore, in present investigation, influence of dynorphin antibodies on trauma induced changes in SCEP was examined in our rat model. In addition, spinal cord edema formation and microvascular permeability disturbances were also investigated. Our results show that intrathecal administration of dynorphin antiserum prior to injury has a beneficial effect on trauma induced electrical activity, microvascular permeability disturbances, and edema formation. These observations indicate that dynorphin is somehow involved in the altered bioelectrical activity of the spinal cord and participates in the pathophysiological processes leading to cell injury.

Neuroscience

Desensitization of glial metabotropic glutamate receptor by agonists exposure

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Metabotropic glutamate receptors are coupled to phospholipase C stimulation and adenylyl cyclase inhibition through G-proteins. C6 glioma cells, that endogenously express the phospholipase C coupled metabotropic glutamate receptor type, were treated with different specific agonists of these receptors and the effect of these treatments on different components of metabotropic glutamate receptor pathway was studied by radioligand binding, phospholipase C activity and RT-PCR assays. Agonists treatment caused a decrease in L-[^{3}H]Glutamate binding to intact cells and membranes in a time dependent manner being maximum at 3–6 hours and recovered at 24–36 hours. This decrease was associated with a significant increase in the mRNA level coding mGluRs. No changes on $G_{q/11}$ mRNA level were detected in any case. However, a significant decrease in L-glutamate stimulated phospholipase C activity was detected after agonist treatments in both membranes and intact cells. This decrease was not associated to significant variations in mRNA level coding phospholipase C β_1 isoform. All these results suggest that agonist exposure causes a desensitisation of glial metabotropic glutamate receptor decreasing not only receptors number but its functionality.

Interaction of hypothalamic nuclei in cardiovascular responses mediated by amino acids

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Dorsomedial hypothalamic (DMH) and paraventricular nuclei (PVN) are two important hypothalamic structures where excitatory and inhibitory amino acids were shown to be involved in the central regulation of cardiovascular responses. In this study the interaction between these two nuclei were investigated by means of microinjection and microdialysis techniques in Sprague-Dawley rats. Steroetaxic surgery was performed by placing intracerebral parenchymal microinjection cannula into the right DMH and microdialysis probe into the left PVN. Iliac artery was also cannulated to monitor the pulsatile blood pressure and heart rate by means of pressure transducer connected to a polygraph Microinjection of 50 pmol NMDA into the DMH was performed and microdialysis perfusates were collected simultaneously from the PVN in conscious rat model. γ-aminobutyric acid (GABA) and L-glutamic acid levels were analyzed by an isocratic HPLC (High Pressure Liquid Chromatography) method with the aid of a fluorescent detector. Microinjection of 50 pmol NMDA into DMH produced significant increases in mean arterial pressure and heart rate. NMDA microinjection into the DMH produced significant increase in L-glutamic acid release in the PVN, but no significant change in GABA release was observed. These results suggest that stimulation of DMH by NMDA results in subsequent stimulation of the PVN.

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Fatty-acid binding proteins in patients with Down syndrome and Alzheimer's disease

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Fatty-acid binding proteins (FABPs) are involved in the intracellular binding, targeting and transport of long-chain fatty acids (FAs) to modulate cell growth and/or differentiation. FABP form a family of proteins displaying tissue-specific expression. The expression of brain type FABP (B-FABP) is spatially and temporally correlated with neuronal differentiation during brain development. Heart type FABP (H-FABP) is widely distributed and present in skeletal muscles, kidney, lung, brain and endothelial cells. It is neuron-specific in postnatal brain and participates in neurite formation and synapse maturation. Epidermal type FABP (E-FABP) is expressed at high levels during neurogenesis, neuronal migration, and terminal differentiation. Although all three FABPs could be involved in normal brain function in prenatal and postnatal life, a neurobiological role of FABPs in neurodegenerative diseases has not been reported yet. These made us evaluate the protein levels of FABPs in brains from patients with Down syndrome (DS) and Alzheimer's disease (AD) and fetal cerebral cortex with DS using two-dimensional (2-D) gel electrophoresis with subsequent matrix-assisted laser desorption ionization mass spectroscopy (MALDI-MS) identification and specific software for quantification of proteins. In fetal brain, B-FABP and E-FABP levels were comparable between control and DS. In adult brain, B-FABP was significantly increased in occipital cortex of DS, and H-FABP was significantly decreased in DS (frontal, occipital, parietal cortices) and AD (frontal, temporal, occipital and parietal cortices). We conclude that aberrant expression of FABPs, especially H-FABP in neurodegenerative diseases could be involved in impaired neurite outgrowth and synapse maturation.

The effects of GABA-A agonist THIP on ethanol self-administration in spontaneously hypertensive rats

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In our previous paper, it was shown that GABA-A receptor antagonist picrotoxin suppressed ETOH (ethanol) self-administration. Recently, several authors indicated that systemic injection of dopamine or serotonine agonists reduced ethanol drinking in rats. Therefore, in the present study we investigated the effects of THIP (4,5,6,7-tetrahydroizokasazolo, 5,4-c pyridin-3-ol) GABA-A receptor agonist in naive and long-term ethanol-experienced rats on ETOH self-administration and on cardiovascular system. Adult 13-17-week-old male, normotensive Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR) were used. Naive rats were examined according to Smith method. Long-term ethanol-experienced rats were studied according to Boyle method. THIP was injected in naive rats at a dose of 8 and 16 mg/kg i.p.

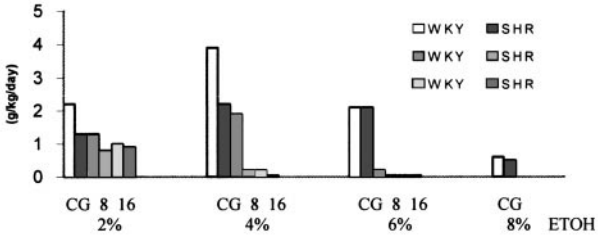

Fig. 1. Influence of THIP on ETOH self-administration in naive SHR and WKY rats

Table 1

Strain	ETOH 2%–10%	ETOH 10%	ETOH 10% + $THIP_{16}$
WKY	3.2 ± 0.5	1.4 ± 0.2	1.4 ± 0.4 (one day)
SHR	2.0 ± 0.2	1.4 ± 0.2	0

Boyle method. Data are means ± S.E.M (g/kg/day)

and in long-term ethanol-experienced rats only at a dose 16 mg/kg i.p. Control group (CG) received saline 3 ml/kg i.p.

Results: As can be seen in Fig. 1 and Table 1 the lower consumption of ethanol in SHR in comparison to WKY rats was observed. Systemic injection of THIP decreased dose-dependently ETOH intake in naive rats of both strains. This effect was more pronounced in SHR (Fig. 1). Similar phenomenon was observed after THIP injection in long-term ethanol-experienced rats. There were no effect on systolic blood pressure and heart rate after THIP treatment.

Activation of ligand- and voltage-gated ion channel by protein and amino-acid metabolites could underlie their neurotoxic effect in uremia

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Increased neuronal excitability may underlie some of the neurological complications in uremic patients. In an effort to identify candidate neuroexcitatory compounds, 17 different uremic retention solutes, including several amino acids and amino acid derivatives, were applied to mouse spinal cord neurons in primary dissociated cell cultures. Using the tight-seal whole-cell technique, a few of the candidate toxins were shown to evoke whole-cell currents in cells clamped at −60 mV. In a first survey, each of the solutes was briefly applied in a concentration of 5 mM. Significant inward whole-cell currents were evoked by guanidinosuccinate, spermine, and 3-indoxyl sulfate, whereas phenol evoked an outward current. Further experiments indicated that guanidinosuccinate-evoked whole-cell currents were due to activation of NMDA-type glutamate receptors in concentrations similar to those found in uremic patients. High (mM) concentrations of spermine activated voltage-gated calcium channels, whereas low (μM) concentrations were found to potentiate guanidinosuccinate-evoked currents through its action on the NMDA receptor-associated polyamine binding site. Whole-cell currents evoked by 3-indoxyl sulfate or phenol seemed to be due to complex interaction with several different ion channels. We conclude that guanidinosuccinate-evoked NMDA receptor activation, possibly potentiated by the neuroexcitatory effects of polyamines and other putative uremic neurotoxins, could be an important mechanism underlying the increased neuroexcitability in uremic brain.

Glutamine transport in C6 glioma cells: Adaptation to the absence of glutamine in the culture medium

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Glutamine (Gln) is one of the key metabolites in the CNS (energy metabolite, precursor of neurotransmitter amino acids, end product of ammonia detoxication, osmolyte), and as such is a routine supplement of CNS cell culture media. C6 glioma cells relatively easily adapt to culturing in a Gln-deprived medium. The present study investigated the effects of Gln deprivation on the characteristics of the different systems that mediate Gln cell membrane transport in the cells. In contrast to a variety of CNS and non-CNS cells, the absence of Gln did not derepress the methyl-amino-isobutyric acid (MeAiB)-sensitive ("system A-dependent") uptake. System ASC became relatively more-, and system N less active than in cells grown in the presence of Gln, but the ion – and substrate specificity of the uptake remained unaltered. System ASC in C6 cells grown in a Gln-supplemented medium shows two features distinct from most other cell types: a) strong pH sensitivity and b) partial tolerance of lithium substitution, pointing to domination of system ASCT2 – an ASC variant strongly expressed in cultured astrocytes. Cells grown in Gln-deprived medium lost lithium tolerance, but not pH-dependence of the uptake, their properties thus resembling system GlnT (SAT1), a neuron-specific variant of system A. By contrast, transport of threonine, a standard ASC system substrate, was not affected by Gln deprivation and showed neither pH dependence nor lithium tolerance, which is typical of an ASC in all the non-CNS tissues.

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GABA inhibits the secretory activity of oxytocinergic, but not vasopressinergic, neurons in conscious rats during emotional stress

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The classical the hypothalamic-neurohypophysial system (HNS) is comprised of neurons originating within the supraoptic nucleus (SON) which project to the neurohypophysis to release the nonapeptides oxytocin (OXT) and vasopressin into the blood after appropriate stimulation. Previous experiments have shown that a single social defeat experience triggers the release of OXT from somata and dendrites into the extracellular fluid of the SON, but not from axon terminals in the neurohypophysis. To further investigate the regulatory mechanisms underlying this dissociated release, we exposed male Wistar rats to a 30-min social defeat experience and monitored the release of the inhibitory amino acids gamma amino butyric acid (GABA) and taurine into the SON using microdialysis. Social defeat caused a significant increase of the intra-SON

release of both, GABA and taurine (up to 480%; $p < 0.01$ vs. pre-stress basal release). To reveal the physiological significance of the intrahypothalamically released GABA – bicuculline, a specific $GABA_A$-receptor antagonist – was administered into the SON by retrodialysis. This treatment increased significantly the release of OXT both within the SON (200%; $p < 0.05$ vs. pre-stress basal release) and – as measured via chronically implanted jugular venous catheters – into blood under basal and stress conditions (up to 200%; $p < 0.01$ vs. pre-stress basal release). However, bicuculline did not affect plasma vasopressin. These data demonstrate that GABA is released within the SON during social defeat to act as an inhibitor of both, central and peripheral OXT secretion during emotional stress. The mechanism described here contributes to the regulatory capacity of the HNS to ensure the appropriate involvement of OXT in the stress response of the animal (supported by DFG, EN 366-21).

β-Amyloid precursor protein, ETS-2 and collagen alpha 1 (VI) chain precursor, encoded on chromosome 21, are not overexpressed in fetal Down syndrome: further evidence against gene dosage effect

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Down syndrome (DS) is the most common human chromosomal abnormality caused by an extra copy of chromosome 21 and characterized clinically by somatic anomalies, mental retardation and precocious dementia. The phenotype of DS is thought to result from overexpression of a gene or genes located on the triplicated chromosome or chromosome region. Reports that challenge this notion, however, have been published. To add to this body of evidence, the expression of β-amyloid precursor protein (APP), ETS-2 and collagen α1 (VI) chain precursor, encoded on chromosome 21, was investigated in fetal brain by western blot and two-dimensional electrophoresis (2-DE). Western blot detected APP and ETS-2 that migrated at ~75 and 50 kDa, respectively. Subsequent densitometric analysis of APP and ETS-2 immunoreactivity did not produce any significant change between controls and DS. Since the metabolic fate of APP determines the propensity of amyloid β production, the expression of the secreted forms of APP (sAPP) had been examined. Neither the expression of sAPPα nor sAPPβ showed any detectable changes among the two groups. Collagen α1 (VI) chain precursor, a protein resolved as a single spot on 2D gel was identified by matrix associated laser desorption ionization mass spectroscopy. Quantitative analysis of this spot using the 2D Image Master software revealed a significant decrease in fetal DS ($P < 0.01$) compared to controls. Linear regression analysis did not show any correlation between protein levels and age. The current data suggest that overexpression per se can not fully explain the DS phenotype.

Unaltered expression of Fas (CD95/APO-1), Caspase-3, Bcl-2 and Annexins in brains of fetal Down syndrome: evidence against increased apoptosis

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Apoptosis is the mechanism by which cells are programmed to die under a wide range of physiological and developmental stimuli. Accumulating evidence indicates that enhanced apoptosis (programmed cell death) in Down syndrome (DS) may play a role in mental retardation and precocious neurodegeneration of the Alzheimer-type. In this regard, alteration of several apoptosis related proteins have been reported in adult DS brain. Fetal DS neurons exhibited increased reactive oxygen species leading to early apoptosis, however, expression of apoptosis related proteins in fetal DS, has never been considered. To address this issue, we investigated the expression of proteins involved in apoptosis including Fas (CD95, APO-1), caspase-3, Bcl-2 and annexins in the cerebral cortex of control and DS fetal brain by western blot and two dimensional electrophoresis. Here, we report that no detectable changes were obtained in fetal DS brain in the expression of Fas, caspase-3, Bcl-2 and Annexins (I, II, V, and VI) compared to controls. In parallel experiment, we also examined the expression of neuron specific enolase (NSE), a neuronal marker found to be decreased in adult DS brain, to see if there is any neuronal loss and no difference was observed between the two groups. Protein expression did not correlate with age. The unchanged levels of Fas, Bcl-2 and annexins together with unaltered caspase-3 expression, a predominant caspase that executes apoptosis in the developing nervous system, suggest that enhanced apoptosis may not be apparent in fetal DS brain as demonstrated for adult DS brain.

Amino acid liberation in brain ischemia: Comparison between experimental ischemia and human stroke

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Introduction. Among the various metabolites indicating neuronal damage, amino acids are regarded particularly important. Detection of amino acids by microdialysis is currently introduced as a neuromonitoring tool in patient care. Here, we present changes in the extracellular concentrations of various amino acids in stroke patients and in experimental stroke in cats.

Method. Cat focal ischemia was produced by occlusion of the middle cerebral artery (MCA) for 1 h followed by 2 h reperfusion. Glutamate, aspartate, GABA, taurine, glycine, serine, glutamine, methionine, threonine, tyrosine, asparagine, valine, phenylanaine, isoleucine and leucine were sampled by microdialysis in the ischemic core and subsequently analyzed by HPLC.

Human microdialysis was performed in patients with large MCA infarction. The microdialysis probes were inserted into primarily non-infarcted tissue in the border zone of the ischemic territory.

Results. Transmitter amino acids rose immediately after occlusion in the cat model. Correspondingly, these substances

increased sharply in the human brain, when the tissue around the probes became infarcted, as shown by positron emission tomography (PET) and CT scan.

In contrast, structural amino acids did not show marked increases or even decreased during severe ischemia in both, experimental ischemia and stroke patients. These substances did increase, however, when the brain tissue was only slightly ischemic, i.e. after reperfusion of the cat brain, when brain swelling occurred, or in human brain, when tissue did not show any infarction in the CT scan but hypoperfusion in the PET image.

Conclusion. Extracellular amino acids detected by microdialysis can serve as markers for secondary ischemia. Severe ischemia is reflected by rapid increases of transmitter amino acids, due to various mechanisms including synaptic release and reversal of reuptake systems. Oligemia seems to be reflected by slow increases of structural amino acids, possibly due to a reduction in cerebral protein synthesis.

Alteration of caspases and other apoptosis regulatory proteins in Down syndrome

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Apoptosis has been implicated in the selective neuronal loss of Down syndrome (DS). Apoptosis activates a family of cysteine proteases with specificity for aspartic acid residues referred to as, caspases that play a key role in dismantling a cell committed to die. Caspases activity is regulated by a variety of proteins that possess a domain resembling the prodomains of caspases. Little is known, however, about the changes of caspases and their regulatory proteins in DS. Here, we investigated levels of nine such different proteins by western blot technique in frontal cortex and cerebellum of control and DS subjects. The protein levels of DFF45 (DNA fragmentation factor 45), and FLIP (FADD like interleukin-1β-converting enzyme inhibitory proteins) were significantly decreased whereas that of RICK (RIP-like interacting CLARP kinase) increased in both regions of DS. In contrast, cytochrome c, Apaf-1 (apoptosis protease activating factor-1), procaspase-9 and ARC (apoptosis repressor with caspase recruitment domain) were unchanged. Procaspase-3 and -8 were significantly decreased in frontal cortex but no significant change was observed in cerebellum. Regression analysis revealed no correlation between postmortem interval and levels of the investigated proteins. However, inconsistent correlation was found between age and levels of proteins as well as amongst the density of individual proteins.

These findings demonstrate that dysregulation of apoptotic proteins does exist in DS brain and may underlie the neuropathology of DS. The study further suggests that apoptosis in DS may occur via the death receptor pathway independent of cytochrome c. Hence, therapeutic strategies that target caspase activation may prove useful in combating neuronal loss in this disorder.

Effects of nitric oxide synthase inhibition on ischemia-induced release of amino acids from rat hippocampus

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In order to examine the differential roles of nitric oxide (NO) induced by either endothelial NO synthase (eNOS) or neuronal NO synthase (nNOS) after transient cerebral ischemia, we investigated the effects of the relatively selective cNOS inhibitor, L-N^5-(1-iminoethyl)ornithine (L-NIO), the relatively selective nNOS inhibitor, 7-nitroindazole (7-NI) and the NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide (PTIO) on hippocampal dysfunction caused by cerebral ischemia. We measured NO concentration, mean arterial blood pressure (MABP), hippocampal blood flow, direct current potential, CA1 population spike (PS) and release of amino acids from rat hippocampus after transient forebrain ischemia, which was induced by 4-vessel occlusion for 10 min. L-NIO (20 mg/kg), 7-NI (25 mg/kg) and PTIO (1 mg/kg) were administered intraperitoncally 20 min before ischemia. PTIO, 7-NI and L-NIO reduced ischemia-induced NO production in the hippocampus during the early period of reperfusion. The rank order of inhibitory potency was PTIO > 7-NI > L-NIO. L-NIO, but not 7-NI, reduced hippocampal blood flow during ischemia and increased MABP before, during and after ischemia, compared with the vehicle group. PTIO increased MABP during and after ischemia. PTIO and 7-NI, but not L-NIO, reduced amplitude of anoxic depolarization induced by ischemia. 7-NI recovered in part PS amplitude 60 min after ischemia. 7-NI, but not L-NIO, reduced ischemia-induced release of aspartate and glutamate, but not taurine. The present study provides further evidence for the idea that in the early stages of transient forebrain ischemia, eNOS-derived NO has a neuroprotective effect in the hippocampus, while nNOS-derived NO has a neurotoxic effect.

The estrogen affects brain protein synthesis in ovariectomized female rats

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The purpose of this study was to determine whether 17-β-estradiol affected the rate of brain protein synthesis in ovariectomized female rats. Experiments were conducted on three groups of 12 wk old female rats: group 1. ovariectomized to reduce the level of plasma estradiol; group 2. ovariectomized and treated with estradiol; and group 3. sham-operated control. The fractional rates of protein synthesis in brain of ovariectomized rats treated with estradiol were significantly greater than in ovariectomized rats without estradiol treatment. In brain, the RNA activity [g protein synthesized/(g RNA□d)] significantly correlated with the fractional rate of protein synthesis. The RNA concentration (mg RNA/g protein) was not related to the fractional rate of protein synthesis in any organ. The results suggest that estrogen treatment of ovariectomized female rats is likely to increase the rate of protein synthesis in the brain, and that RNA activity is at least partly related to the fractional rate of brain protein synthesis.

Chemistry, pharmacology and clinical studies of novel antidepressant peptides

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We have synthesized a series of new peptides that have demonstrated potent antidepressant activity in animal models

for depression and in Phase IIA and IIB clinical trials. MIF-1 (Prolyl-Leucyl-Glycinamide) an endogenous brain peptide has been reported to have some clinical activity in patients with unipolar depression with few apparent side effects. We have undertaken a study to determine the effect of molecular structural changes on the antidepressant activity of this peptide. We evaluated our new derivatives in a stress-induced animal model for depression, i.e. Porsolt Test, We have found that 4-F-Phe-4-OH-Pro-Arg-Gly-Trp-NH_2 (INN 00835) is superior in all the statistical parameters used. In comparative testing INN 00835 was more active than Prozac (fluoxetine) and Zoloft (sertraline) in our antidepressant model. A U.S. patent has been granted on these compounds.

The clinical results of INN 00835 show that it is effective in over 80% of depressed subjects when blood levels exceeded therapeutic threshold with no significant side effects. INN 00835 has a rapid onset of action, 3–5 days (vs. 2–6 weeks for currently marketed products) with sustained effects for months following 5 to 10 doses over 1–2 weeks.

Origin and regulation of extracellular D-serine in the rat brain: an in vivo microdialysis study

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Using an in vivo dialysis technique, we have studied the extracellular contents of endogenous free D-serine in comparison with that of L-serine, glycine and L-glutamate in the brain of the freely moving rat. A high amount of D-serine was detected in the dialysate obtained from the medial prefrontal cortex and striatum, whereas the cerebellar dialysate contained only a trace concentration of the D-amino acid. Intra-medial prefrontal cortex perfusion of a sodium channel activator, veratrine, augmented the extracellular release of glycine and L-glutamate but a slight decrease in that of D-serine in a tetrodotoxin-sensitive manner in the prefrontal area. Moreover, selective destruction of neuronal cell bodies in the medial frontal region by means of local infusion of an excitotoxin quinolinate resulted in a marked reduction of extracellular and tissue levels of D-serine in the infused prefrontal region. These findings suggest that endogenous D-serine might be liberated into the extracellular space from non-neuronal cells or certain exceptional neuronal cells probably by a carrier-mediated process in the mammalian prefrontal cortex. Also, the endogenous D-amino acid has been indicated to be accumulated or synthesized, at least in part, in the neuronal cells.

Decreased protein levels of nucleoside diphosphate kinase A/Nm23-H1 in patients with Down syndrome and Alzheimer's disease

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Nucleoside diphosphate kinase (NDPK) catalyzes a transfer of the terminal phosphate from nucleoside triphosphates ((d)NTPs) to nucleoside diphosphates ((d)NDPs) and has been suggested to be involved in the regulation of wide variety of cellular functions. In addition, NDPK isoforms (A and B) are encoded by nm23 genes (H1 and H2), which are related with the metastatic potential of some tumors. Although NDPK/nm23 has been also implicated to modulate neuronal cell proliferation, differentiation and neurite outgrowth, a neurobiological role of NDPK/nm23 in neurodegenerative diseases has not been reported yet. Here we evaluated the protein levels of NDPK-A/nm23-H1 in brains from patients with Down syndrome (DS) and Alzheimer's disease (AD) using two-dimensional (2-D) gel electrophoresis with subsequent matrix-assisted laser desorption ionization mass spectroscopy (MALDI-MS) identification and specific software for quantification of proteins. NDPK-A/nm23-H1 was significantly decreased in brain regions (frontal, occipital, parietal cortices) of both DS and AD compared to controls. We conclude that the down-regulated NDPK-A/nm23-H1 upon neurodegeneration could play a pivotal role in a wide range of neurobiological functions such as neurite outgrowth and consequently these could result in functional disturbance of the nervous system in DS and AD.

Brain α-endosulfine is manifold decreased in brains from patients with Alzheimer's disease: a tentative marker? and drug target?

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α-Endosulfine has the sulfonylurea-like ability to block ATP-sensitive potassium (K_{ATP}) channels and is expressed in a wide range of tissues. Although the blockade of K_{ATP} channels has been reported to be involved in the release of neurotransmitters, the neurobiological role of α-endosulfine has not been studied yet. We examined the levels of α-endosulfine protein in frontal cortex and cerebellum from patients with Alzheimer's disease (AD). α-Endosulfine was extremely decreased in both regions of AD compared to controls. This could result in the continuous opening of K_{ATP} channels with subsequent decrease of neurotransmitters release and change of potassium fluxes. This study is of great significance for providing a neurobiological function of α-endosulfine in brain and furthermore, α-endosulfine could serve as a useful marker for the diagnosis of AD and a target for drug treatment.

D-2-Hydroxyglutarate: characterization of a novel endogenous neurotoxin

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D-2-Hydroxyglutaric aciduria is an inherited neurometabolic disorder of unknown etiology characterized by progressive neurodegeneration of vulnerable brain regions during infancy and early childhood, resulting in psychomotor retardation, hypotonia, seizures, macrocephaly, enlarged lateral ventricles, delayed cerebral maturation as frequent neurological presentation in affected children. The disease is biochemically characterized by the accumulation of D-2-hydroxyglutarate (D-2), which is structurally similar to L-glutamate (= 2-amino-glutarate). We therefore investigated in primary neuronal cultures from chick and rat, whether D-2 induces excitotoxic neuronal damage. Here we report that D-2 decreased cell viability in a concentration- and time-dependent fashion by disturbance of intracellular calcium homeostasis as determined by fura-2 measurement. Furthermore, fluorescence microscopy using dihydrorhodamine-123 revealed an increased generation of reactive oxygen species (ROS) elicited by expo-

sure to D-2. *N*-methyl-D-aspartate (NMDA) receptor blockade specifically prevented excitotoxic neuronal damage as well as increased calcium influx and ROS production, suggesting that D-2 is an agonist at NMDA receptors. Patch-clamp investigation confirmed that D-2 activated recombinant NMDA receptors in HEK293 cells. Furthermore, activity measurement of single respiratory chain complexes revealed a specific inhibition of complex V activity by D-2. We conclude that excitoxic mechanisms contribute to the neuropathology of D-2-hydroxyglutaric aciduria, highlighting new neuroprotective strategies for this neurometabolic diseases.

Effect of chronic treatment with imipramine on the potency of zinc to inhibit [^{3}H]MK-801 binding to NMDA receptor

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We have demonstrated previously that chronic antidepressant treatment, which is required for clinical improvement, reduced the function of the glutamate/NMDA receptor complex. On the other hand antidepressant-like properties of antagonists of the NMDA receptors have been demonstrated. Zinc is an inhibitor of the NMDA receptor activity. Like other NMDA receptor antagonists, zinc exhibits antidepressant-like effects in rodent screening tests. In the present study we examined the effect of chronic treatment with imipramine on the potency of zinc to inhibit [^{3}H]MK-801 binding to NMDA receptor in the mouse and rat hippocampus and cerebral cortex. Chronic treatment with imipramine produced significant increase in the potency of zinc to inhibit [^{3}H]MK-801 binding in mice cerebral cortex but not in rats. No significant changes in the potency of zinc to inhibit [^{3}H]MK-801 binding were found after chronic imipramine treatment in hippocampus in both species. The present data are in agreement with previously reported antidepressant-induced reduction of the NMDA receptor function and suggest that effect of imipramine on the zinc and NMDA receptor interaction is species-specific.

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The effects of aging and diet restriction on the expression of NMDA receptor subunits

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These studies were designed to determine the effects of aging and an aging intervention on NMDA subunit expression. *In situ* hybridization and receptor autoradiography were performed on naïve or behaviorally-tested C57Bl/6 mice of different ages (3, 10–15, and 26–30 months old) and diet groups (*ad lib*-fed and diet restricted). There were age-related decreases in both e2 and z1 mRNA density in naïve, *ad lib*-fed mice. Correlations were found between changes in e2 subunit mRNA and agonist binding and z1 mRNA expression and antagonist binding. Diet restriction significantly improved learning ability, slightly spared glutamate binding to NMDA sites and improved z1 mRNA expression in older mice. Significant correlations were found between agonist binding and both learning ability and e2 and e1 mRNA density. Learning ability in the old mice also correlated with the ratios of mRNA expression for e1 and e2 and/or z1 subunits. These results suggest that changes in NMDA receptor binding and the relationship between subunit expression levels are important for maintaining memory functions in older animals.

The effect of hyperforin on amino acid neurotransmitter release from mouse cortical slices

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Extracts of St John's Wort (Hypericum perforatum L.) are widely prescribed for the treatment of mild to moderate depression and the putative antidepressant constituent is probably hyperforin. In this study the effect of hyperforin was investigated on the release of neurotransmitter amino acids. Coronal cortical slices (400 mm) were cut and perfused with gassed (95% O2, 5% CO2) aCSF at 37°C. Two-minute samples of perfusate were collected and aspartate and glutamate were assayed by HPLC. Potassium- and veratridine-stimulated release was elicited by administering 2 pulses of K+ (60 mM) or veratridine (20 mM) 30 minutes apart.

In control experiments the second K+ pulse elicited glutamate release which was 80% of the first pulse. Hyperforin (5 mM) perfused for 30 minutes prior to, and during, the second K+ pulse significantly increased glutamate release to 170% ($P < 0.001$, $n = 6–8$). Release elicited by the second veratridine pulse was 70% of the first pulse for both glutamate and aspartate. Hyperforin (5 mM) increased this release to the second pulse to 160% and 130% respectively ($P < 0.001$, $n = 6–8$). When perfused on its own for 30 minutes, hyperforin (5 mM) increased the basal release of glutamate ($P < 0.001$, $n = 4–5$).

In conclusion, the increase in the release of neurotransmitter amino acids observed following hyperforin is possibly mediated through a facilitatory action on voltage-operated Ca2+ or Na+ channels.

Characterization of NMDA receptor glycine/glutamate allosteric interactions in rodent and human brain

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N-methyl-D-aspartate (NMDA) receptors are ligand gated ion channels widely distributed in mammalian brain, which play a crucial role in important physiological mechanisms, such as excitatory transmission, neuronal migration and memory formation. A peculiar feature of NMDA receptors is the absolute requirement of L-glutamic acid and glycine for the opening of the channel. Noteworthy, these two aminoacids reciprocally modulate binding at their respective recognition sites. Aim of this work was to study NMDA receptor glycine/glutamate interactions in rat and human brain.

Binding of the NMDA antagonist [^{3}H]CGP39653 to rat cerebral cortical membranes was inhibited by glycine. The overall effect of glycine consisted in a decrease of [^{3}H]CGP39653 affinity, with a parallel increase of the receptor affinity for glutamate. The glycine antagonist GV150526A competitively reversed glycine inhibition, proving that the modulation was via the glycine binding site.

[^{3}H]CGP39653 binding to rat brain sections revealed the existence of regionally distinct NMDA receptor subtypes with difference glycine/glutamate interactions. In most regions of the human brain NMDA receptors presented the same allosteric modulation of [^{3}H]CGP39653 binding, as revealed by large section autoradiography technique. Nevertheless, detection of any regional variation was not possible, probably due to the high intersubject variability.

Adaptive changes on NMDA receptor function and expression in rat cultured cortical neurones

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The effect of long-term high K^+-treatment on neuronal survival, cellular maturation, NMDA receptor (NR) splice variant expression, and receptor function was investigated in primary cultures of rat cortical neurones.

Long-term incubation (up to 15 days) with 25 mM K^+ significantly increased neuronal survival and induced multiple morphological changes associated with promoted cellular maturation. Cultures grown in medium containing 25 mM K^+ also exhibited multiple changes in NR1 splice variant expression according to RT-PCR studies performed with primer pairs flanking the alternatively spliced regions, in order to estimate the ratios of the corresponding 3′ and 5′ splice variants. NR1-1 and NR1-3 (each containing exon 21) were decreased, whereas NR1-2 and NR1-4 (each lacking exon 21) were increased, accordingly. The predominant expression of NR1-b was further increased. After administration of TTX, each of the K^+-induced changes on mRNA expression was virtually abolished. In voltage-clamp recordings (holding potential: −70 mV), NMDA induced inward currents in a concentration-dependent manner with a maximum effect of −745 pA under control conditions. Neurones treated with 25 mM K^+ showed a significantly diminished response to NMDA (max. response: −368 pA). In conclusion, the present data indicate that a sustained increase in neuronal activity induces adaptive changes in NR1 splice variant expression and a decrease in receptor function. Thus, alternative splicing associated with a diminished receptor-cytoskeletal linkage may be important compensatory mechanism in preventing cellular damage due to long-term activation of excitatory NR. It seems conceivable that this mechanism contributes to the promoting effects of 25 mM K^+ on neuronal survival and maturation.

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Decreased level of APG-2, a member of the heat shock protein 110 family, in murine brain after kainate administration

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There is accumulating reports that kainate-induced seizures elicit expression of various heat-shock proteins (HSPs) in the brain, such as HSP27, HSP32, and HSP70. However, no investigation has been carried out on changes in level of APG-2, a member of HSP 110 family, after excitatory amino acid-induced seizures. By means of an immunoblot assay, we determined the levels of HSP70 and APG-2 in discrete brain structures of mice after a single intraperitoneal injection of kainate or NMDA. APG-2 level was significantly decreased in the frontal cortex, hippocampus, and striatum 3 days after kainate administration, while HSP70 level was increased in these regions following the administration. Decreased level of APG-2 returned to the control levels in the three regions 10 days after kainate administration. No significant changes were observed in levels of both HSP70 and APG-2 in hypothalamus, midbrain, medulla-pons, and cerebellum of kainate-treated mice. By contrast, NMDA administration did not significantly affect both levels in any of the regions examined. These results suggest that, unlike the case of HSP70, APG-2 expression could be temporarily down regulated by signals peculiar to kainate, but not by those peculiar to NMDA, in murine telencephalon.

Effects of intracerebroventricular injection of glucagon like peptide-1 and its related peptides on levels of amino acids in the rat hypothalamus

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High concentrations of glucagon-like peptide-1 (7-36) amide (GLP-1) and its specific receptor (GLP-1R) have been found in the rat hypothalamus. In this study the actions of GLP-1 and its related peptides, exendin-4 (GLP-1R agonist), exendin (9-39) (GLP-1R antagonist) and GLP-1(9-36)amide (major GLP-1 metabolite) on levels of amino acids (Glu, Asp, Gln, Gly, Tyr, Trp, GABA) in the hypothalamus were investigated. ^{125}I-GLP-1 binding in rat hypothalamic membranes was competed by the peptides in the following order of potency; GLP-1 > exending-4 > exendin (9-39) > GLP-1(9-36)amide. Intracerebroventricular (ICV) GLP-1 (4 nmoles) produced a statistically significant reduction in levels of all measured amino acids compared with saline injected controls, whereas 4 nmoles of exendin (9-39) was ineffective. Exendin-4 produced a statistically significant reduction in the levels of Trp, Glu, and Tyr. GLP-1(9-36)amide showed a statistically significant increase in the level all the amino acids tested in this study. Prior administration of exendin (9-39) or GLP-1(9-36)amide blocked the effects of GLP-1 on the levels of the amino acids. These data are consistent with exendin-4 being a GLP-1R agonist and exendin (9-39) being a specific GLP-1R antagonist. GLP-1(9-36)amide, a primary metabolite of GLP-1, appears to act as an endogenous antagonist at the GLP-1R.

Role of cholecystokinin$_A$ and cholecystokinin$_B$ receptors in anxiety

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Cholecystokinin (CCK), a family of neuropeptides, seems to be involved in anxiety. Evidence from several laboratories indicates that the ansiogenic effects of CCK are mediated by CCK_B receptors. However it has been reported that CCK_A receptors have been found in brain and CCK_A receptor antagonists have ansiolytic properties. The aim of this work was to study whether or not CCK_A receptors are also involved in the modulation of anxiety. Male rats were cannulated in the lateral ventricle and CCK (9 fmol) and/or CCK antagonists (900 fmol) were injected 7 days after surgery. Anxiety was evaluated in the elevated plus-maze test and locomotion in an open-field test.

Ansiogenic effects were observed when CCK_B receptor agonists (CCK8nS; CCK4) or a mixed CCK_B and CCK_A receptor agonist (CCK8S) were injected. In contrast, CCK33, a CCK_A receptor agonist or CCK_{1-21} and CCK_{26-29} were uneffective. Furthermore, the ansiogenic effects of CCK8S

were prevented by the previous (15 min) administration of L365,260 (CCK_B receptor antagonist) but not by devazepide (CCK_A receptor antagonist). No effects on locomotion were observed in any condition. These results indicate that CCK_A receptors are not involved in anxiety, as measured by the elevated plus-maze test.

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The anti-epileptic, valproate, may potentiate the induction of neural tube defects by inhibiting serine hydroxymethyltransferase and/or the glycine cleavage system

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Congenital conditions (i.e. neural tube defects: NTG) have a multifactorial aetiology. Deficiencies in the folate and transsulfuration pathways have, in recent years, been positively linked to NTD and other dysmorhogenic syndromes. Efficient one-carbon metabolism is crucial for the synthesis of DNA precursors, the remethylation of homocysteine and biomethylation of DNA. More than 80% of the one-carbon units that flow through the metabolic system in mammals and birds are derived from L-serine and glycine, the natural substrates for SHMT. The mitochondrial glycine cleavage enzyme system (GCES) can potentially compete with SHMT for tetrahydrofolate (THF) in the generation of the methylenetetrahydrofolate pool. Valproate (Depakene, Epilim), an anti-epileptic agent, appears to be strongly associated with hyperhomocysteinemia, several other induced metabolic conditions, the inhibition of the GCES and an increased incidence of NTD in epileptic women of child-bearing age. The exact mechanisms of valproate-induced NTD are not yet clear. We investigated the association of the teratogenic properties of valproate with the inhibition of SHMT and/or the GCES in developing embryos.

Chicken embryos were treated with sodium valproate (VPA) and pregnant female mice (C57BL) received intraperitoneal injections of VPA, during the critical period of embryonic neural tube development. Control embryos were treated with sterilised saline solution. Harvested embryos were subsequently investigated for congenital abnormalities and hepatic SHMT and GCES activities quantified with radiometric assays. The effect of VPA on hepatic DNA synthesis was monitored (^{3}H-thymidine incorporation into embryonic DNA) and the DNA-methylation status determined (DNA N^5-methylcytosine levels). Dose-responsive incidences of NTD were observed in VPA treated embryos. Very few defects occurred in control embryos. SHMT and GCES appeared to be inhibited in liver extracts of VPA-treated embryos. Hepatic DNA synthesis was significantly compromised and 5-MC levels were altered in VPA-treated embryos. The inhibition of either SHMT and/or GCES activities appeared to be associated with valproate-induced NTD in the chicken and mouse embryo models. The primary mechanism of this effect can probably be ascribed to a restriction in the flow of one-carbon units through the metabolic system, decreased synthesis of DNA precursors and alterations in the methylation status of DNA.

Interactions dopamine-glutamate in the cognitive and rewarding effects of ethanol

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Ethanol is long known to cause dose-related biphasic effects and we recently found that ethanol bidirectionally affects also working memory. The euphoriant and excitatory effects produced at low doses are associated with the rewarding action of ethanol and are thought to be mediated by the activation of the mesolimbic dopamine (DA) system. However, ethanol monophasically stimulates mesolimbic DA release in the nucleus accumbens, even at doses that cause hypnosis and coma. In contrast, ethanol biphasically modulates mesocortical DA release in the prefrontal cortex (PFC). The changes in DA release induced by ethanol are time locked with corresponding changes in extracellular glutamate levels. These biphasic effects of ethanol on PFC DA and glutamate are matched by biphasic changes in the performance in a spatial delayed alternation task – a working memory test that is sensitive to proper function of the PFC – suggesting a link between DA and glutamate transmission in the cognitive effects of ethanol. Focal application in the PFC of the competitive AMPA/kainate receptor antagonist CNQX suppresses both DA release and the improvement of working memory induced by low doses of ethanol. These results suggest that ethanol may increase DA transmission in the PFC and enhance working memory functions by increasing the release of glutamate, thereby stimulating non-NMDA glutamate receptors. The enhancing effect on working memory by low, excitatory doses of ethanol may be perceived as rewarding and could constitute an important neurobiological mechanism for excessive ethanol drinking.

The role of glutamate and aspartate on the epileptic process

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Glutamate and asparate are considered as the main excitatory neurotransmitters in brain and spinal cord, in addition to their role in energy metabolism, synthesis of proteins and detoxification of ammonia.

Glutamate and aspartate are centrally involved in basic mechanisms generating epileptic seizures and in epileptogenesis. Stimulated release of glutamate and aspartate was detected in vivo and in vitro following neuronal depolarization. Photic stimuli has increased glutamate release from visual cortex, and afferent brachial stimulation has increased the endogeneous release of glutamate from contra-lateral sensorimotor cortex compared to ipsi-lateral side. Similar results were achieved after local application of Tityustoxin or Veratridine to the sensorimotor cortex.

Implantation of cobalt powder over the right sensorimotor cortex of rats produced an epileptogenic lesions characterized by contra-lateral fore and hind limb jerks and an increase in the frequency of EEG spikes. The jerks started after 6 days with maximum myoclonic activity (15 jerks/min). The concentration of glutamate in the epileptogenic focus was decreased significantly by 29% ($P < 0.01$) compared to the non-epileptogenic area on the left sensorimotor cortex, which was dissected but not treated with cobalt. Part of the decrease in glutamate could be related to the enhancement of in-vivo release from the epileptogenic lesion to the extra-cellular fluid.

Kindling is the best model for studying the development of the epileptic focus (Epileptogenesis), it could be achieved by repeated intra-cerebral micro-injection of glutamate (1.5 μmol), aspartate (0.75 μmol) or NMDA (2–4 nmol), or repeated electrical stimulations of specific brain regions. In addition, glutamate antagonists particularly those specifically acting on the NMDA receptor type e.g. 2-amino-5-phosphonovaleric acid (AP5) and 2-amino-7-phosphonopheptanoic acid (AP7) have been found to inhibit seizures in epileptic animals and inhibit the development of electrically kindled epilepsy.

Pre-synaptic glutamate receptor agonists like (1S, 3S)-ACPD the agonist of group II, and L-AP_4 the agonist of group III receptors has reduced Ca^{++} uptake and glutamate release, thus it has inhibited epileptogenesis by preventing the increase in both seizure score and after-discharge duration. Injection into fully kindled animals has produced an anti-epileptic effect by reducing the mean seizure score and by increasing the mean generalized seizure thresholds. This results suggest the mechanism by which pre-synaptically active glutamate receptor agonists block the development of the chronically epileptic state induced by electrical kinding, and indicate that their anti-convulsive activity is due to inhibition of pre-synaptic glutamate and/or asparate release following blockade of pre-synaptic Ca^{++} entry.

Testing the changes in glutamate release from hyperactive brain tissues, and the effect of different glutamate agonists and antagonists, supports the role of glutamate in initiating the process of epileptogenesis, and contributes in developing new anti-epileptic agents.

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Roles of chloride ion and divalent cations in GABA transport

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The functional roles of Cl(−) and divalent cations in the Na(+)/Cl(−)/GABA cotransport were examined in *Xenopus* oocytes expressing the human GAT-1 (hGAT-1) GABA transporter cDNA.

Our results showed that Cl(−) was not absolutely required for Na(+)/GABA transport via the hGAT-1 (Loo et al., *J Biol. Chem.* **275**:37414–37422, 2000). The Cl(−) interacted with the transporter to modulate the binding of external Na(+). Although hGAT-1 transported Cl(−) across the membrane with a stoichiometry of 2Na(+):1Cl(−):1GABA, the transported Cl(−) did not contribute to the net charge translocated across the membrane, suggesting a Cl(−)/Cl(−) exchange mechanism during the GABA transport cycle.

The GABA transport via the hGAT-1 is also modulated by divalent cations. The uptake of [3H]-GABA was inhibited significantly when both Ca(2+) and Mg(2+) were removed from the uptake buffer. Several divalent cations tested were individually able to sustain the GABA uptake. In contrast to uptake, the GABA efflux was enhanced significantly upon removal of both Ca(2+) and Mg(2+) from the efflux buffer. The GABA transporter inhibitor SKF89976A blocked the enhanced efflux, suggesting that the hGAT-1 operated faster in the reverse mode in the absence of external divalent cations. These results suggest a regulatory role for the divalent cations in GABA transport.

Role of alpha5 containing $GABA_A$ receptors in hippocampal synaptic function

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The role of alpha5 containing $GABA_A$ receptors in hippocampal synaptic function has been investigated using pharmacological and electrophysiological techniques, as well as following disruption of the alpha5 subunit gene in knockout mice (KO). In the CA1 region of the hippocampus the induction of long-term potentiation (LTP) is powerfully regulated by GABA mediated synaptic currents (IPSCs). Agents that inhibit GABA-mediated transmission potentiate LTP induction, whereas allosteric agonists such as benzodiazepine-site agonists which slow the decay kinetics of IPSCs suppress LTP induction. In alpha5 KO mice paired pulse facilitation of the amplitude of excitatory synaptic potentials is selectively enhanced in the CA1 region but not dentate gyrus. Likewise, the frequency and rise time of spontaneous IPSCs were similar in WT and KO slices. However their amplitude was significantly smaller in KO mice. Furthermore, a significantly greater proportion of IPSCs were best fitted to a mono exponential function in KO mice compared to WT animals. Thus alpha5 containing $GABA_A$ receptors contribute to functional postsynaptic receptors on CA1 pyramidal cells in the hippocampus and modulate a post-synaptic component of synaptic facilitation.

Effect of GABA derivatives in a model chronic stress in male rats

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The purpose of the study is to investigate effect of phenil (karphedon, mephebut, gammoxin) and circle (pyracetam) GABA derivatives on reproductive function of stressed male rats.

The adult male rats were stressed by immobilization exposure (2 hours) twice in week during 6 weeks. Four from five groups of stressed males were given substances (daily) at doses: karphedon – 50 mg/kg, mephebut – 10 mg/kg, gammoxin – 40 mg/kg, pyracetam – 200 mg/kg. The treated males were mated with intact females during 12 days. After the mate the treated males and more in 10 days all the mated females were sacrificed and investigated.

Analysis of our data indicates that the time of spermatozoa motion and epididymal sperm counts were decreased 73.2% ($P \leq 0.05$) and 49.1% ($P \leq 0.05$) respectively when compared with their intact controls. GABA derivatives have a softening effect on functional parameters of spermatozoa stressed males. Karphedon and pyracetam increased the time of motion spermatozoa 86.3% ($P \leq 0.05$), karphedon and mephebut drew near sperm counts to intact control level. The result of mate show that pregnancy rate was increased ($P \leq 0.05$) by stress exposure and pregnancy rate of females mated with GABA stressed males was some more ($P \geq 0.05$) than that of intact controls. The general embryonic morality was increased twice by stress and so the number of embryos was reduced 48.8%. The GABA derivatives exposure to stressed male rats reduced the embryonic mortality of their posterity and increased the number of embryos to intact control level.

Our findings demonstrate that GABA derivatives administration has a protective effect on reproductive function of stressed male.

Effect of MK-801 (NMDA receptor antagonist) applied during pregnancy on the central dopamine receptors reactivity in adult offspring rats

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The amino acids glutamate and aspartate, and perhaps certain of the analogs, mediate most of the excitatory synaptic

transmission on the brain. The realization that glutamatergic pathways are involved in such diverse processes in epilepsy, ischemic brain damage and Parkinsons' disease, is of a great practical interest. There are at least three functional classes of ionotropic glutamate receptors: N-Methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5 methyl-4-isoxazolepropionic acid (AMPA) and kainate (KA). Other central neurotransmitter systems are under NMDA influence. Some data point on neuroprotective action of NMDA antagonist on nigrostriatal pathway. In the present study female Wistar rats were exposed during pregnancy with daily injected MK-801 (dizocilpine) 0.5 mg/kg SC. Control rats received tap water only. Behaviour of 3 month old male offsprings was investigated by several psychopharmacological methods. Oral activity, yawning, locomotor activity, stereotypy and catalepsy were recorded following respective central dopamine receptors agonists and antagonists administration (SKF 38393, quinpirole, apomorphine, haloperidol). Our results indicate that MK-801 applied during pregnancy modulate reactivity of the central dopamine receptors in adult offspring rats.

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The expression and role of glutamate receptors in trigeminal neurons during the ontogeny of mastication

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The development of mammalian ingestive behavior is characterized by a transition from suckling to chewing, two distinct motor behaviors. We hypothesize that this transition is accompanied by changes in brainstem circuitry underlying these movements. Since glutamatergic neurotransmission is critical for the proper functioning of brainstem circuitry responsible for mastication, we investigated the development of glutamate receptors in trigeminal motoneurons (Mo5) and mesencephalic trigeminal neurons (Me5); neurons comprising the circuitry responsible for jaw movements. We conducted a series of receptor immunohistochemistry experiments that characterized the expression of iontotropic and metabotropic glutamate receptors (mGluRs) during early postnatal development. The functional roles of NMDA, AMPA and mGluRs in neonatal Mo5 were investigated using in vitro electrophysiological experiments. Results demonstrated that the spatial and temporal expression of AMPA, NMDA and group I and II mGluRs are developmentally regulated within and between Mo5 and Me5 during early development. Electrophysiological data demonstrate that mGluRs function pre- and postsynaptically to modulate synaptic transmission between trigeminal premotoneurons and Mo5. Furthermore, NMDA induced bursting is developmentally regulated and coincident with the transition from suckling to chewing behaviors. Our studies suggest that the transition from suckling to chewing is accompanied by changes in the composition and function of glutamate receptors.

Fetal life in Down Syndrome starts with normal neuronal density but impaired dendritic spines and synaptosomal structure

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Information on fetal brain in Down Syndrome (DS) is limited and there are only few histological, mainly anecdotal reports and no systematic study on the wiring of the brain in early prenatal life exist. Histological methods are also hampered by inherent problems of morphometry of neuronal structures. It was therefore the aim of the study to evaluate neuronal loss, synaptic structures and dendritic spines in the fetus with Down Syndrome as compared to controls by biochemical measurements. 2 dimensional electrophoresis with subsequent mass spectroscopical identification of spots and their quantification with specific software was selected. This technique identifies proteins unambiguously and concomitantly on the same gel. Fetal cortex samples were taken at autopsy with low postmortem time, homogenized and neuron specific enolase (NSE) determined as a marker for neuronal density, the synaptosomal associated proteins alpha SNAP [soluble N-ethylmaleimide-sensitive fusion (NSF) attachment protein], beta SNAP, SNAP 25 and the channel associated protein of synapse 110 (chapsyn 110) as markers for synaptosomal structures and drebrin (DRB) as marker for dendritic spines.

NSE, chapsyn 110 and beta SNAP were comparable in the control fetus panel and in Down Syndrome fetuses.

Drebrin was significantly and remarkably reduced and not even detectable in several Down Syndrome brain samples.

Quantification of SNAP 25 revealed significantly reduced values in DS cortex and alpha SNAP was only present in half of the DS individuals.

We conclude that at the time point of about 19 weeks of gestation (early second trimester) no neuronal loss can be detected but drebrin, a marker for dendritic spines and synaptosomal associated proteins alpha SNAP and SNAP 25 were significantly reduced indicating impaired synaptogenesis. Early dendritic deterioration maybe leading to the degeneration of the dendritic tree and arborization, which is a hallmark of Down Syndrome from infancy.

Aberrant expression of dihydropyrimidinase related proteins-2, -3 and -4 in fetal Down Syndrome brain

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Pathfinding of growing axons to reach their target during brain development is a subtle process needed to build up contacts between neurons. Abnormalities in brain development in Down Syndrome (DS) are described in a couple of morphological reports but the molecular mechanisms underlying abnormal wiring in fetal DS brain are not yet elucidated. We therefore performed a study using the proteomic approach to show differences in protein levels involved in the guidance of axons between control and DS brain in early prenatal life. Proteins obtained from autopsy of human fetal abortus were applied on 2-dimensional gel, identified and quantified. We quantified 5 members of the semaphorin/collapsin family, the

dihydropyrimidinase related proteins 1–4 and the collapsin response mediator protein-5 (CRMP-5) in 8 DS and 7 control cortex samples.

DRP-1 and CRMP-5 levels were comparable in the control and DS samples.

Evaluation of DRP-2, DRP-3 and DRP-4 revealed significantly decreased levels of 2 of the 15 spots assigned to DRP-2 and increased levels of one spot assigned to DRP-3 and increased DRP-4 in DS brain.

We conclude that as early as from the 19th week of gestation pathfinding cues of the outgrowing axons are impaired in DS. These findings may help to elucidate mechanisms leading to abnormalities in neural migration of DS brain.

NMDA receptors and cyclooxygenase as targets for neuroprotection from the cytotoxicity of inflammatory proteins

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Inflammatory processes play an important role in the degeneration of basal forebrain cholinergic cells Alzheimer's disease. The proinflammagen lipopolysaccharide (LPS) was infused chronically into the basal forebrain of young rats. We then determined whether the administration of two novel non-steroidal anti-inflammatory drugs or a pancaspase synthesis inhibitor, zVAD, could provide neuroprotection from the cytotoxic effects of the neuroinflammation. We also determined whether the administration of the non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist, memantine, could provide neuroprotection from the cytotoxic effects of the neuroinflammation. Chronic LPS infusions decreased choline acetyltransferase activity and increased the number of activated microglia within the basal forebrain. Caspases 3, 8 and 9 activity was increased in ventral caudate/putamen. Non-steroidal anti-inflammatory drug therapy attenuated the toxicity of the inflammation upon cholinergic cells and reduced caspases 3, 8, and 9 activity in the caudate/putamen. zVAD significantly decreased the levels of caspases 3, 8 and 9 but did not provide neuroprotection for cholinergic neurons. Memantine significantly attenuated the cytotoxic effects of chronic inflammation upon cholinergic cells. These results suggest that prostaglandins contribute to the degeneration of forebrain cholinergic neurons in Alzheimer's disease and that the cytotoxic effects of prostaglandins occur upstream to NMDA receptor activation.

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Amnestic effects of microinjection of NMDA receptor antagonist AP5 to the goldfish telencephalon

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Intracranial administration of N-methyl-D-aspartate (NMDA) receptor antagonists block learning of classical and avoidance conditioning in goldfish. Studies with goldfish have shown that NMDA receptors are mostly dense in the telencephalon and telencephalon ablation impairs avoidance learning. The present study investigated amnestic effects of microinjection of NMDA receptor antagonist AP5 to the goldfish telencephalon in avoidance conditioning. In Experiment 1, fish received no injection or microinjections of saline or various doses of AP5 to their telencephalon 20 minutes before three semiweekly training sessions. Fish were tested without injections in Session 4. A one-way ANOVA with multiple comparisons on the test scores showed that AP5 produced anterograde amnesia in a dose-dependent manner. In Experiment 2, fish received several training sessions and a microinjection of various doses of AP5 20 minutes before testing. The test scores showed that AP5 did not decrease avoidance responses, suggesting that microinjection of AP5 did not impair performance processes. In Experiment 3, fish received microinjections of AP5 or saline to their telencephalon immediately following three semiweekly training sessions and were tested without injections in Session 4. A one-way ANOVA on the test scores showed that AP5 did not produce retrograde amnesia.

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Tryptophan modulates striatal serotonergic activity relative to fatigue

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We have been reported that mechanism of fatigue in the brain relates to enhanced extracellular tryptophan and serotonergic function. Brain concentration of tryptophan is not only dependent on the change of tryptophan which originates from the centarl nervous system, but also enhance tryptophan entering the brain from the blood-brain barrier and peripheral circulating tryptophan which is a trigger. Supplementation of L-tryptophan (2 uM) into the incubation medium with the synaptosomal striatum causes tryptophan to the extrasynaptosomal release by high K+ stimulation. Injecting L-tryptophan (1 mM/30 min) into the left striatum by microdialysis method can induce early fatigue for running time of rats. On the other hand, tryptophan deficiency rats (body weight average 200 g) were made by tryptophan free feeding for 2 weeks, and the rat's running time increased (>100 min difference).

These results suggests that tryptophan is a potent active substance for fatigue in the brain. The active zone may be presynaptic terminal and the tryptophan itself may be releasing neuromodulators.

(We appreciate that tryptophan free diet was provided by AJINOMOTO CO., INC., Japan.)

Uptake and release of D-serine in rat brain synaptosomes

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Our recent studies on the distribution of free D-serine, together with the D-serine action on the glycine site of the NMDA type glutamate receptor, suggest that the D-serine can be an endogenous modulator of the NMDA receptor. To explore the possible removal systems for brain D-serine signaling, we have evaluated the uptake of [3H]D-serine into the synaptosomal P2 fraction from the rat cerebral cortex. The cortical P2 fraction was able to accumulate [3H]D-serine in a temperature- and pH-dependent and saturable manner. The kinetic analysis indicates that cortical D-serine transport occurs by an apparent single-component system with Km value of 283 μM and a Vmax value of 207 pmol/mg protein/min. Depletion of Na^+ and Cl^- ions remarkably decreased D-serine uptake into the cortical P2

fraction. The pharmacological profile of the inhibition of D-serine uptake by various amino acids was different from those of glycine uptake system and other amino acid transporters reported. D-serine uptake activity was preferentially observed in the brain tissues such as cerebral cortex and cerebellum to the peripheral tissues. The present data support the view that the endogenous D-serine is taken up mainly through a carrier-mediated transport system to regulate the extracellular concentration in the mammalian brain.

D-arginine, L-canavanine and naloxone reversed antinociception elicited by L-arginine, L-ornitine, L-citruline in rats

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The mammalian brain contains all the urea cycle intermediates, whereas enzymes participating in the conversion of L-ornithine (L-Orn) into L-citrulline (L-Cit) are absent, resulting in an incomplete urea cycle. The discovery of nitric oxide (NO) synthase that catalyses the formation of NO and L-citrulline as a co-product from L-arginine (L-Arg) in the brain has indicated an additional pathway for L-Arg metabolism. L-canavanine (L-Cav), is a potent antimetabolite and structural analog of L-arginine, produced by legumes such as the jack bean, *Canavalia Ensiformis*. L-Canaline (L-Can) is a potent inhibitor of ornithine aminotransferase.

Our previous results indicated that L-Cav, L-Cit, L-Arg, and L-Orn exerted an antinociceptive effect, whereas L-Canaline induced hyperalgesia in rat. L-Canavanine exert stronger antinociceptive effect than L-Arginine, L-Ornithine and L-Citrulline.

The aim of the present study was to investigate are D-Arg, L-Cav and Naloxone reversed the analdesic effects of L-ornithine, L-citrulline and L-arginine. The experiments were carried out on male Wistar rats. The changes in the mechanical nociceptive threshold of the rats were measured by the Radall-Selitto paw pressure test using and analgesimeter (Ugo Basile). The amino acids were applied intracerebroventricularly (i.c.v.) at a dose 20 μg/rat.

The present results shown that D-Arg, L-Cav and Naloxone reversed antinociception.

Plant Amino Acids

The regulation of lysine metabolism in cereal crops

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A major nutritional drawback of cereal seeds is a deficiency in some amino acids, in particular lysine. Biochemical, molecular and genetic studies have considerably increased our knowledge concerning the regulation of the aspartate pathway, by which lysine is synthesized. Among the enzymes involved in lysine metabolism, aspartate kinase (AK) and dihydrodipicolinate synthase (DHDPS) control the regulation of lysine biosynthesis, whereas lysine: 2-oxoglutarate reductase (LOR) and saccharopine dehydrogenase (SDH), have been shown to play a key role in the breakdown of lysine. In general, lysine overproduction can be obtained by altering the sensitivity of DHDPS to lysine, but accumulation of this amino acid in cereal seeds requires further manipulation of LOR and/or SDH. This suggestion is strongly supported by five main points: (1) Cereal mutant or transgenic plants do not exhibit any significant accumulation of lysine in seeds, but only in other tissues. (2) The enzymes of lysine degradation, LOR and SDH, are endosperm specific in cereals only. (3) The opaque-2 mutant, which exhibits higher concentration of soluble lysine and protein lysine in the seed, contains several-fold lower LOR and 2-fold lower SDH activity when compared to the wild-type maize. This reduction in activity in the opaque-2 mutant is due to a reduced protein LOR-SDH concentration by reduction of the *ZLKRSDH* gene transcript. Furthermore, the *opaque*-2 maize gene has been shown to regulate AK and LOR activity. (4) Intermediates of lysine catabolism accumulated in the seeds of soybean and canola lysine overproducing plants, suggesting the presence of reduced LOR and/or SDH activities. (5) Among cereals and although still below the recommend values by FAO, rice exhibits the higher concentration of lysine, but LOR and SDH are present in much lower activities. Also, in *Phaseolus vulgaris*, LOR and SDH activities were shown to be around 10-fold lower then in maize endosperm. The regulation of the LOR activity is complex and involves a calcium dependent phosphorylation/dephosphorylation mechanism. It remains to be seen whether this latter mechanism can be controlled, so as to allow the production of more crop plants that contain elevated concentrations of lysine in the seed.

The genetic progress for NUE can be accelerated with the use of secondary traits that possess high inheritance and correlation with productivity. Several traits have been studied such as chlorophyll concentration, plant height, leaf senescence, anthesis-silking interval, kernel number, activities of enzymes of N assimilation and loci of quantitative traits for assisted selection.

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Cadmium effect on antioxidant enzymes in higher plants

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Cadmium (Cd) is a toxic element that normally occurs in low concentrations in soils, however in areas that have been subjected to mining the concentration can be high, varying from 100–600 mg/Kg dry weight. In addition, following the aplication of sewage sludge to agricultural land, Cd can accumulate in the topsoil. Although Cd is not an essential nutrient for plants, the metal ion is taken up rapidly by the roots and on most occasions causes inhibition of growth. However a number of plants

(termed hyperaccumulators) that grow on metalliferous soils, are able to translocate Cd from the roots and accumulate it in high concentrations in the shoots. Cd may be detoxified in plants by combination with a family of sulphur rich peptides termed phytochelatins. Cd has the capacity to inhibit a range of enzyme activities in plants, in particular those of the Calvin cycle and chlorophyll biosynthesis. Evidence that Cd causes the production of reactive oxygen species (ROS) has also been obtained.

We have investigated the antioxidant responses of radish, soybean and sugarcane to Cd treatment. Seedlings were grown in increasing concentrations of $CdCl_2$, ranging from 0.01–1 mM, for up to 96 h in a hydroponic system. Analysis of Cd uptake indicated that most of the Cd accumulated in the roots, but some was also translocated and accumulated in the leaves. Roots and leaves were analysed for catalase (CAT), glutathione reductase (GR) and superoxide dismutase (SOD) activities. GR activity increased considerably in the roots of all plant species tested after exposure to the metal, indicating a direct correlation with Cd accumulation. CAT activity also increased in roots but to a much lesser extent when compared to GR and also varied depending upon the plant species. The analysis of native PAGE enzyme activity staining, revealed several SOD isoenzymes in leaves of all plant species, however, only in radish was a clear increase in activity observed. The results suggest that in these plants, the activity of antioxidant enzymes responds to Cd treatment. The main response may be via the activation of the ascorbate-glutathione cycle for the removal of hydrogen peroxide, or to ensure the availability of glutathione for the synthesis of Cd-binding proteins.

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Metabolism and accumulation of biogenic amines in plant food

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All plant cells, tissues and organs provide the biosynthetic machinery and capacity to synthesise aliphatic polyamines. However, in physiological conditions only some organs and tissues synthesise polyamines, such as apical buds and sprouts, root apex, lateral buds of branches and secondary roots, as well as superficial layers of young stems and leaves, like epidermis, subepidermis and parenchyma cells. Apical roots can also synthesise polyamines, but these activities in physiological conditions are lower than that of the shoots. This patterns recalls the one of auxins. Polyamines are accumulated in high concentrations in storage organs, such as seeds, but not in tubers like *Helianthus tuberosus*, potato or tuberised roots such as the carrot. Also some fruit, e.g. oranges, contain high level of free polyamines, putrescine in particular. All other organs obtain polyamines through translocation via phloem tubes and xylem vessels.

In plants, in addition to free polyamines, many polyamines are conjugated to hydroxycinnamic acids, the hydroxycinnamic amines, that only rarely represented outside the plant kingdom. This compounds are paticularly abundant in Solanaceae family, where they can represent as much as 90% of the total polyamine pool, but they can be detected in different concentrations in many other families.

The role of free and conjugated polyamines and their importance in food is discussed.

Long-term accumulation of amino acids and amines in stressed cereals and its reversal by alkanolamine treatments

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Drought, salinity or other environmental stressors promote the accumulation of free amino acids, amines and other organic N-metabolites with low molecular weight.

In this contribution the influence of drought on the accumulation of amino acids, polyamines and trigonelline in growing barley plants and barley grains was examined.

In comparison to non-stressed plants we obtained in stressed plants, exposed to drought before flowering, a higher concentration of proline (increase: 4-fold), N-trimethylglycine (2-fold), histidine (3-fold), tryptophane (1,7-fold), putrescine (1,4-fold), spermine (1,7-fold) and trigonelline (2-fold) in the dry matter of barley sprouts. In addition to this, drought caused an increase of the N-content in the plant biomass (35%) as a result of growth inhibition (34%).

Six weeks later the content of soluble N-metabolites and protein was analyzed in non-stressed and pre-stressed barley plants again. During this reproductive period of plant development all the test groups were cultivated under the same moisture conditions. The analysis of N-metabolites in the ripening grains showed, surprisingly an after-effect of the drought stress. For example, in grains of pre-stressed barley the concentrations of free proline, histidine, tryptophane and Asx+Glx were threefold to fivefold higher than in grains of non-stressed barley.

Depending on the resistance of barley cultivars to drought the biochemical response was different: In plants with low resistance the increase of amino acids and amines was higher than in resistant cultivars. However, resistant cultivars have already high genuine concentrations of N-metabolities in non-stressed plants.

By treatments with choline or 2-aminoethanol the stress-promoted accumulation of amino acids and trigonelline was diminished.

Consequently, different biochemical responses of cereals to drought result in changes of product quality and nitrogen use.

Increasing lysine in corn

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Our goal is to increase the lysine content in corn. We have used genetic engineering to increase lysine synthesis and to prevent metabolic breakdown of lysine. To increase synthesis we circumvented the normal feedback control of a key enzyme in the lysine biosynthetic pathway, dihydrodipicolinic acid synthase (DHDPS). Lysine-feedback-insensitive DHDPS, encoded by the Corynebacterium dapA gene, was expressed from seed-specific promoters in transformed corn seeds. Expression of DHDPS in the corn embryo, but not in the corn endosperm, resulted in a 20 to 30-fold increase in the accumulation of free lysine in the seeds and the total seed lysine content nearly doubled.

Lysine breakdown products have been observed in transgenic seeds that accumulate high levels of free lysine. We isolated a corn gene for the bifunctional enzyme lysine ketoglutarate reductase (LKR)/saccharopine dehydrogenase (SDH), which catalyzes the first two steps in lysine breakdown. Knockout of LKR/SDH in corn by either mutation or genetic engineering results in a 10-fold increase in seed free lysine. Combination of feedback-insensitive DHDPS with knockout of LKR/SDH results in 2 to 3-fold higher levels of free lysine than

DHDPS alone. No adverse effects on seed or plant agronomic performance are associated with the high lysine trait.

Regulation by oxidative stress of 5′-adenylylsulfate reductase, an enzyme in the cysteine synthesis pathway of plants

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5′-adenylylsulfate (APS) reductase catalyzes a key reaction in the plant sulfate assimilation pathway leasing to the synthesis of cysteine and the antioxidant glutathione. In *Arabidopsis thaliana* APS reductase is encoded by a family of 3 genes. *In vitro* studies revealed that the enzyme product derived from one of the APS reductase genes (APR1) is activated by oxidation, probably through the formation of a disulfide bond. Redox titrations show that the regulation site has a midpoint potential of −327 mV at pH 8.5 and involves a 2-electron redox reaction. Exposure of a variety of plants to ozone induces a rapid increase in APS reductase activity that correlates with the oxidation of the glutathione pool and is followed by an increase in free cysteine and total glutathione. During the response to ozone the level of immuno-detectable APS reductase enzyme does not increase. Treatment of *A. thaliana* seedlings with oxidized glutathione or paraquat induces APS reductase activity even when transcription or translation is blocked with inhibitors. The results suggest that a post-translational mechanism controls APS reductase. A model is proposed whereby redox regulation of APS reductase provides a rapidly responding, self-regulating mechanism to control the glutathione synthesis necessary to combat oxidative stress.

News from a model fungus: Inducer and the GATA factor AreA are necessary for *in vivo* binding of a pathway-specific transcription factor in *Aspergillus*

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In *Aspergillus nidulans* the structural genes coding for nitrate reductase (*niaD*) and nitrite reductase (*niiA*), share a common promotor region of 1,200 bp. We have previously characterized *in vitro* and *in vivo* the physiologically relevant *cis*-acting elements for the two synergistically acting transcriptional activators, NirA and AreA. We have further shown that AreA is constitutively bound to a central cluster of four GATA sites and is directly involved in opening the chromatin structure over the promoter region and thus making additional cis-acting binding sites accesible. Here we show that the asymmetric mode of NirA-DNA interaction determined *in vitro* is also found *in vivo*. Binding of the NirA transactivator is not constitutive as in other binuclear C_6-Zn^{++}-cluster proteins but depends on nitrate induction and additionally, on the presence of a wild type *areA* allele. Dissecting the role of AreA further, we found that it is required for intracellular nitrate accumulation and therefore could indirectly excert its effect on NirA via inducer exclusion. But in a strain accumulating nitrate independently of AreA NirA binding and chromatin rearrangement is not triggered by nitrate in the absence of AreA.

Engineering of cysteine and methionine biosynthesis in potato

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Higher plants, being a source of reduced sulfur for animal nutrition, assimilate inorganic sulfate into cysteine which is subsequently converted to methionine, another sulfur-containing amino acid. In order to investigate the possible regulatory points of the cysteine and methionine biosynthesis pathway a series of transgenic potato plants was engineered using clones encoding enzymes of the branched pathway from serine to cysteine as a pathway intermediate and from aspartate further on to methionine. Increased cysteine levels were obtained in the leaves of serine acetyltransferase (SAT) sense and cystathionine β-lyase (CbL) antisense transformants. Furthermore, glutathione levels were elevated in SAT plants while downregulation of CbL was desastrous for plant growth, eventually. Increased methionine levels were successfully obtained in potato by antisense inhibition of threonine synthase (TS). Accumulation of free methionine was not only observed in source leaf tissues but as well in tubers. This enzyme competes with cystathionine gamma-synthase for the common substrate O-phosphohomoserine at the branchpoint between threonine and methionine synthesis, respectively. Important control points of the biosynthesis of cysteine and methionine in potato, thus, turned out to be SAT and TS, while further studies on overexpression of cystathionine gamma-synthase, CbL and MS did not reveal any substantial effect on potato methionine biosynthesis.

Free amino acids of plants in the process of phytoremediation

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Amino acids in root exudates of plants may be chelate agents as an alpha-amino acid can act like a bidentate ligand, forming a five-membered heterocyclic ring with suitable metal cations thus increasing mobility of metals.

Recently we have showed that application of growth regulators led to sharp increase of root exudative activity of some cultural (*Zea maize L.*) and wild cereals (*Festuca rubra L.*, *Lollium perenne L.*) during first days of germination. In this work we present results obtained in experiments with *Lollium perenne L.*, grown on sterile sand and on soils contaminated with great quantities of *Zn*.

Detailed analysis of amino acid content of root exudates of several types of maize (hybrid, several lines, an Opaque-2 mutant line) showed that the specie had more certain amino acids (cysteine, aspartic and glutamic acids and their amides, serine) in root exudates than cultural ones. These amino acids has more possibility for chelation due to existance of one more polar or ionogenic functional groop. Seeds of *Lollium perenne L.* were treated with growth regulater and planted on soils contaminated with salts of zink. It was shown that during 15 days of germination quantity of *Zn* in primary leaves increased from 121,46 to 243,75% and decreased in soil: in upper layer from 95,74 to 85,07, midde layer from 95,83 to 82,04, lower layer from 85,72 to 72,74 mkg/kg correspondingly. Thus, it was shown that stimulation of root exudative activity by pretreatment with a growyh regulator may be succesful in cleaning of soils and basicly this is a good method for phytoremediation.

Physiology/Exercise and Sport

EEG-Changes in humans during regeneration after heavy physical strain with the influence of L-theanine; an amino acid in green tea

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The hypothesis: "L-Theanine has relaxing effects of central nervous system of human beings", was verified by electroencephalographical methods.

Methods: 14 male, healthy sport-students, free of drugs or stimulants, participated weekly in a cross-over study. After exhaustive bicycle-ergometer test as an individual, reliable, stress model, the subjects recovered by lying in a segregated shaded room. Three testdrinks with different L-Theanine content (d1 = placebo, d2 = 50 mg, d3 = 200 mg) were given in a randomised, double-blind order. All test-conditions were standardized strictly. EEG-recordings (closed eyes) were carried out (M1 = 3 min. after stress/before testdrink, M2 = 30 min.-, M3 = 45 min.-, M4 = 60 min.-, M5 = 120 min. after testdrink) with the CATEEM® System. Absolute and relative EEG-spectral-power were examined.

Results: Significant reductions in all frequencies (exception theta-power) were found in early recovery, being not significant influenced by testdrinks. Qualitative different behavior trends were found in frontal-, central-, occipital-regions with increased alpha1, theta (frontal) and decreasing beta1 relative-power earlier in recovery with d3. These findings were related to relaxing effects. After ingestion of L-Theanine alpha2-, beta1-power at occipital regions decreased faster (M2) to placebo recovery levels (M3/M4). Thus it may be concluded that L-Theanine has no pharmaceutical effect on the down regulation system but supports the physiological mechanisms during recovery after physical stress in human brain.

Arginine and cysteine in muscle cytosol of rats' heart after exercise, hypoxia or challenge with six selected cardioactive drugs

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Levels of the amino acid L-arginine (a major endogenous donor of nitric oxide-NO), cysteine (sulfur-containing amino acid, important for atriopeptins and endothelins synthesis), and of total free amino acids, were assayed by gas-liquid chromatography in cytosols of rats' atrial and ventricular muscle cardiomyocytes. The tissues were assayed after the rats had been exposed to either exercise (swimming), hypoxia or one of six cardioactive drugs such as propranolol, digoxin, pentylenetetrazol, reserpine, isoproterenol and caffeine. Physical stress and the examined drugs significantly reduced the total amount of cytosolic free amino acids in both cardiac muscles. In the cytosol of the heart atrial muscle, reserpine, propranolol and pentylenetetrazol increased the relative content of L-arginine, while hypoxia and digoxin decreased it. In the cytosol of the ventricular heart muscle, hypoxia and all six drugs used, decreased the relative levels of L-arginine. Hypoxia and isoproterenol exerted the strongest effect. Exercise completely abolished the levels of cysteine in the atrial heart muscle. Propranolol, isoproterenol, caffeine and pentylenetetrazol increased the ratio of cysteine to the total free amino acids in the atrial muscle, while physical stress and all cardioactive drugs tested increased this ratio in the ventricle muscle. Disappearance of cysteine from the heart's atrial muscle after intensive exercise may be attributed to its utilization for atrial natriuretic factor and/or for endothelin synthesis, during stress. On the other hand it seems that hypoxia and isoproterenol are strong stimulants of NO production, and consequently decrease the tissue levels of L-arginine, which is the major endogenous donor of NO acting as the endothelin antagonist.

Homocysteine and ultrascopic nuclear appendages in vitamin B12 deficiency

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Measurement of serum levels of vitamin B12 is a screening test for detection of deficiency of this vitamin but low levels do not always indicate a deficiency of the vitamin. Measurements of serum homocysteine and methylmalonic acid (MMA) are used to confirm this deficiency because two enzymes involved in their metabolism have been shown to require vitamin B12, but these results can also be inaccurate.

Vitamin B12 deficient white cells exhibit ultrascopic nuclear appenages which have been shown to contain DNA; this finding could possibly be used as another confirmatory test of vitamin B12 deficiency. Twenty-seven patients (mean age – 76.6 years) with low serum B12 were studied by electron microscopic determination of the percent of neutrophils exhibiting these appendages and routine clinical parameters.

Only one patient did not have nuclear appendages; the others had a range of 0.8%–17.6% of neutrophils examined. There was a significant correlation of homocysteine ($r = .46$, $p < .05$) and MMA ($r = .53$, $p < .01$) with serum B12 levels but no correlation of appendage number ($r = .18$) with serum B12. There was no correlation of appendage number with homocysteine ($r = .25$) or MMA ($r = .04$).

These results suggest that B12-deficient white cell nuclear appendages do not measure the same metabolic pathways as homocysteine and methylmalonic acid and may be useful in confirmation of vitamin B12 deficiency. Further extensive clinical evalution would be necessary to explore this possibility.

Inhibitory effect of somatostatin on neutral amino acid transport in isolated brain microvessels

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Addition of somatostatin-14 or of some of its analogs was found to cause a selective inhibition, up to 50%, of the uptake of large neutral amino acids by isolated brain microvessels. Although the luminal and abluminal sides of brain endothelial cells are both capable of taking up large neutral amino acids, only the uptake from the abluminal side was apparently inhibited by somatostatin. The involvement of a type-2 somatostatin receptor was suggested by assays with a series of receptor-specific somatostatin agonists, and was confirmed by the inhibition release caused by a specific type-2 receptor antagonist. A type-2 specific mRNA was indeed shown to be present both in bovine brain microvessels *ex vivo* and in primary cultures of endothelial cells from rat brain microvessels.

Hemoglobin is a potential source of bioactive peptides (hemorphins) towards the Renin Angiotensin System

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Hemorphins represent a bioactive peptide class which contents between 4 and 10 amino acids and generated from the proteolysis of an hemoglobin "strategic zone". Many activities have been related to hemorphins such as *in vitro* anti tumour effect, analgesia effects *in vivo*, and a potential role in the Renin Angiotensin System (RAS).

As far as their activity towards the RAS is concerned, it was demonstrated that they could inhibit angiotensin converting enzyme (ACE) and aminopeptidase N activity. So they could reduce angiotensin II formation and angiotensin IV degradation. Moreover some hemorphins, LVV-Hemorphin-7 and VV-hemorphin-7, could behave like angiotensin IV receptor binding competitor. Further it could be interesting to study the angiotensin IV potentiality to interact with ACE.

Inhibition studies showed that it was possible that angiotensin IV could behave like a competitive inhibitor of ACE. So some hemorphins could interact at different RAS steps to inhibit ACE. Additionally to their inhibition of angiotensin I conversion, they could inhibit angiotensin IV degradation and consequently cause ACE feedback inhibition. Inhibition studies have been checked with RAS natural substrate (angiotensin I) and confirmed that angiotensin IV, VV-hemorphin-7 and mainly LVV-Hemorphin-7 could be natural ACE inhibitors. So the hemorphins regulatory role in the RAS appears to be more and more probable.

Effect of methionine on the testicular function of prostate precancerous old male rate

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The role of administration of each of methionine and finastride on the testicular function of both normal and prostate precancerous old male rats was investigated.

For normal animals, neither methionine nor finastride has exerted any significant change in the hormonal profile after teatment for 20 days. However methionine alone could exert a significant change in both testosterone and prostatic specific antigen {PSA} levels after treatment for 40 days.

On the other hand, both methionine and finasteride significantly increased the levels of testosterone and androstenedione, whileas markedly reduced the levels of dihydrotestosterone and prostatic specific antigen {PSA} after treatment of prostate precancerous old male rats for a period of 20 days.

Noteworthy, continuation of treatment for aperiod of 20 days realized marked improvement of hormonal profile of the prostate precancerous old male rats.

Glycine, exercise and fatigue

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Several observations in our department point to some role of glycine in fatigue and exercise. 1) In the framework of a study on the involvement of one-carbon matabolism in patients suffering from a polymorphic episodic psychosis, amino acid loading tests with serine, glycine and alanine were performed. A few hours after the administration of glycine, approximately 40% of the patients reported overwhelming feelings of fatigue and/or showed vegetative symptoms. 2) In patients suffering from chronic fatigue syndrome, we found increased plasma levels of glycine in 20% of the female patients. Moreover, 60–70% of these patients omplained about a distorted sensory perception of objects. 3) Young soccer players were observed during a period of 10 months, while in the course of this period eight blood samples were taken for amino acid analysis. Based on the number and severity of injuries this population was divided into injury-prone and not injury-prone soccer players. It was found that in injury-prone soccer players plasma glycine levels during the whole observation period were significantly lower than in subjects who were not injury-prone.

The consequences of the above mentioned observations will be discussed.

Does L-theanine have an influence on the relaxation after severe physical exercise? Evaluation using electrosympathicography

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50 percent of amino acids in green tea leaves are represented by L-theanine (5-N-ethylglutamine). Previous rat experiments demonstrated effects of L-theanine to act on metabolism of neurotransmitters. It was therefore suggested that is causes the relaxing effects of green tea. To examine its influence as a component of a drink on the sympathetic nervous system after maximal physical exercise skin resistance measurements through electrosympathicography (ESG) were used. After individual maximal exercise on a bicycle-ergometer test-drinks with different amounts of L-theanine (0, 50 and 200 mg) were administered to 14 healthy volunteers in a randomised cross-over double-blind distribution on a weekly base. ESG was monitored before and immediately after exercise as well as 13, 30, 45, 60, 75 and 135 minutes after end of exercise. All test-conditions were standardized strictly. A characteristic ESG-course with subsequent qualities could be shown: 1. Decreasing skin resistances after exercise could be established in each volunteer. 2. ESG-activation levels before exercise could not even be reached again after a period of regeneration of 2¼ hours. 3. Maximal electrodermal activity did not appear immediately after exercise, but after 13 minutes. However, L-theanine could not significantly influence peripheral sympathetic electrodermal activity during the regeneration after maximal physical exercise.

Leucine supplementation during training period and training sessions in male power athletes

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Essential amino acid leucine has many important roles in the body. Therefore the purpose of the present study was to investigate if leucine supplementation has effects on serum amino acid profile and performance following training period or following single training sessions. All experiments were carried out in a randomized double blind cross-over procedure during a training season. Thirty six adult male track and field power athletes served as subjects. In experiment 1 ten of them were given leucine (50 mg/kg body weight per day) as tablets. The concentration of leucine decreased significantly (20%) in the placebo group (P; n = 10) during 5 weeks but not when leucine was taken. Also total amino acids (TAAs) decreased strongly (19%) during 5 weeks when dally protein intake was 1.26 g/kg body weight. In experiment 2 the subjects (n = 16) carried out a single strength training seasion (STS) and consumed a drink containing leucine 100 mg/kg body weight. Following STS leucine in serum increased by 11% (ns) when leucine was taken but decreased strongly (30%) in P, in experiment 3 the subjects (n = 12) underwent at 7 days interval two maximal anaerobic running exercise (MARE) tests on treadmill (n × 20s with a recovery of 100s between the runs) until exhaustion. The subjects consumed drinks containing leucine (200 mg/kg body weight) or placebo 50 min before the test runs. Following MARE the concentration of leucine strongly increased by 28% whereas isoleucine (25%) and valine (15%) strongly decreased with the supplementation but no changes occurred in P. There were no improvements in physical performance either in MARE or in explosive strength (experiment 2) with leucine supplementation. The date suggest that leucine supplementation during a training period and before single training sessions prevents decreases in serum concentration of leucine and may have also effects on some other single amino acids. This may be beneficial during intensive training although improvements in performance were not observed in this study.

Serum amino acid profile following training period and training sessions in male power athletes

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Since there are only limited data regarding effects of training period or training sessions on serum amino acid profile, the purpose of this study was to investigate serum amino acid changes following training period and following three different training sessions. The subjects consisted of 31 track and field adult male power athletes. In experiment 1 eleven of them performed a 5-week training period including a training sessions per week, which included sprint work, speed endurance work, endurance work, weight training, and jumps. Significant decreases in the fasting concentrations of total amino acids (TAAs) (19%), branched chain amino acids (BCAAs) (21%), essential amino acids (EAAs) (19%) and leucine (20%) were observed following training with the daily protein intake of 1.26 g/kg body weight. In experiment 2 eleven subjects performed a short run session (SRS) of 3 × 4 × 60 m with recoveries of 120 s and 360 s, and a long run session (LRS) of 20 s runs with recoveries of 100 s until exhaustion. There were no significant changes in TAAs following the sessions but BCAAs decreased by 8% in SRS and by 7% in LRS. Leucine decreased by 10% following SRS but only by 5% (ns) following LRS. The peak blood lactate concentrations after SRS and LRS were 13.8 ± 1.9 mmol/L and 16.4 ± 1.3 mmol/L, respectively. In experiment 3 sixteen subjects carried out a strength training session (STS), which consisted of jumps and heavy resistance exercises (speed and maximum strength) during 90 minutes. The TAAs decreased significantly by 14%, BCAAs by 24% and leucine by 30% following STS, while the peak blood lactate concentration was 2.2 ± 0.4 mmol/L. These data indicate that remarkable decreases occur in the concentration of amino acids during a training period with the daily protein intake of 1.26 g/kg body weight. The decreases in serum amino acids are more pronounced following a strength training session than following lactic anaerobic running sessions.

Glutamine acts as a multipurpose regulator of amino acid and peptide transport across the blood-brain barrier

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Isolated brain microvessels, the in vitro equivalent of the blood-brain barrier, have distinct Na^+-independent uptake systems for the uptake of large hydrophobic amino acids, of enkephalins and of deltorphins, as shown by the absence of reciprocal inhibition. Both D- and L-glutamine were capable, if added to the extracellular buffer, of exerting a competitive inhibition on the uptake of all these substrates. A trans-stimulatory effect was instead induced, in all cases, by L-glutamine preloading of the microvessels – the D-stereoisomer being instead ineffective, probably because of only L-glutamine could be taken up, in a concentrative manner, by some Na^+-dependent concentrative system(s). All the Na^+-independent systems present in brain microvessels seem therefore to share some structural feature responsible for their common susceptibility to interference by L-glutamine. This amino acid, whose synthesis can take place in the astrocytes, in the pericytes and also in the endothelial cells of the microvessels, plays a critical role in regulating the movements of several different substrates across the blood-brain barrier.

Two new cross-linking amino acids having pyridine skeleton in elastin: Implication of ammonia in biosynthesis

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Isolation and structure analysis of two amino acids from bovine ligamentum nuchae elastin hydrolysates revealed the presence of pyridine cross-links in elastin. The structures of these amino acids were determined to have 3,4,5- and 2,3,5-trisubstituted pyridine skeletons both with three carboxylic acids and a mass of 396 ($C_{18}H_{28}N_4O_6$), identified as 4-(4-amino-4-carboxybutyl)-3,5-di-(3-amino-3-carboxypropyl)-pyridine and 2-(4-amino-4-carboxybutyl)-3,5-di-(3-amino-3-carboxypropyl)-pyridine. We have named these pyridine cross-links, desmopyridine (DESP) and isodesmopyridine (IDP), respectively. Structure analysis of these pyridine cross-links implied that the formation of these cross-links involved the condensation reaction between ammonia and allysine. The elastin incubated with ammonium chloride showed DESP and IDP levels increased as the allysine content decreased. DESP and IDP were measured by HPLC with UV detection and were

found in a variety of bovine tissues. The DESP/desmosine and IDP/isodesmosine ratios in aorta elastin were higher than in other tissues. DESP and IDP contents in human aorta elastin were found to be gradually increased with age. The concentration of IDP was significantly elevated in aorta elastin of rat with chronic liver cirrhosis induced by carbon tetrachloride when compared with normal rats.

The effect of *in vivo* and *in vitro* glutamine supplementation on human neutrophils

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The provision of glutamine to marathon runners has resulted in a decreased, self-reported incidence of illness. Increasing evidence – *in vitro*; and *in vivo* suggests that neutrophils in humans may benefit from exogenous glutamine. The provision of glutamine *in vivo* should replete the marked decrease in the blood concentration observed after stress such as clinical trauma or prolonged, strenuous exercise. Beneficial effects of glutamine supplementation include increased phagocytic activity and reactive oxygen intermediate production *in vitro*; decreased neutrophilia and IL-8 production (a chemoattractant for neutrophils) *in vivo* and *ex vivo*. The aim of the present study was to establish whether glutamine supplementation *in vitro* and *in vivo* affects neutrophil function at rest and after exhaustive exercise. In addition, it was planned to establish the presence of glutaminase in human neutrophils, which has not yet been achieved, although glutaminase is present in rat neutrophils.

Methods: Blood samples were taken from marathon runners receiving either glutamine or placebo, immediately after and one hour after a race. Measurements included the plasma concentration of glutamine (enzymatic assay), IL-8 production (ELISA), and neutrophil activity. The latter was measured with two different techniques for measuring oxidative burst in whole blood, one of which was a novel chemiluminescence assay (Knight Scientific Ltd, U.K.) with the fluorescent label, Pholasin, and two different stimuli, f-Met-Leu-Phe (FMLP) and phorbol-myristate-acetate (PMA). In addition, isolated whole cells and subcellular neutrophil fractions were assayed for the presence of glutaminase.

Results: The plasma glutamine concentration was reduced overall by 26% 1hr after the race ($p < 0.02$). There was an apparent decrease (only close to significance, $p < 0.13$) in IL-8 production in the glutamine group compared with the placebo group. Neutrophil function did not change between groups at any stage. The incidence of illness was 46% higher in the placebo group than the glutamine group in the week after the race. Neutrophils from four out of six subjects gave an increased response (39.2%) to fMLP when incubated with glutamine compared with no glutamine, and four out of four gave an increased response to PMA (31.1%). In the fMLP experiments there were two individuals who did not respond to the addition of glutamine. However, the response was not diminished whether or not glutamine was present. In separate studies, the effect of glutamine on lipopolysaccharide-induced IL-8 production was also monitored.

Conclusions: The provision of glutamine after prolonged, exhaustive exercise appears to modify exercise-induced neutrophilia via a reduction in IL-8 production and to reduce the incidence of illness in the following week. *In vitro* data suggest a role for glutamine in neutrophil metabolism. Disappointingly, little or no evidence of the presence of glutaminase was found in human neutrophils. The three different methods used, freeze-thaw, homogenisation, nebulisation were apparently not sufficient to break open the granules. Current studies are addressing this problem.

Supplementation of amino acids in sport

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It is essential that the developing muscle has adequate amino acids for the synthesis of actin and myosin as well as those required for a multitude of enzymes involved in muscle metabolism. With carbohydrates and lipids, the body is able to store a reserve as glycogen and triglycerides respectively; however this is not the case with amino acids Creatine supplementation in increasingly being used as a dietary supplement by athletes during high intensity, short term exercise to improve physical performance since it is converted in the muscle to phosphocreatine. Transporters which permit creatine to cross the muscle membrane namely CRT1 and CRT2 (a Na^+ and Cl' dependent mechanism) have now been identified. Creatine uptake is enhanced by the ingestion of carbohydrate at the same time as supplementary creatine. This may be due to increased circulating levels of insulin or insulin-like growth factor 1.

More recently attention has been focussed upon the various transporters for amino acids across the muscle membrane. Certain criteria are needed for the amino acids to enter the blood which include the presence of specific carriers for its transport across cells of the gastrointestinal tract, such as enterocytes, as well as minimal metabolism within these cells. A wide number of different transporters has been identified, which include neutral amino acids and cationic amino acids. Despite the evidence which suggests that supplementation with some amino acids can influence metabolism, and therefore athletic performance, much more experimental work is still required in this area.

Correlations between central nervous parameters and hormonal regulations during recovery from physical stress are influenced by L-theanine

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In animal studies L-theanine was shown to influence neurotransmitter systems. Thus it may be helpful in managing stress regulation. So we observed the down regulation after physical stress in the brain (measured by EEG-mapping) and in peripheral hormonal systems (plasma levels of catecholamines, cortisole, prolactine, serotonine, measured by HPLC). N = 14 healthy students consumed drinks containing either 0, 50 or 200 mg L-theanine in randomized double-blind trials in the min 6–14 after a near maximal bicycle step test. Measurements were done directly after exercise (**M1**) and 30 (**M2**), 45 (**M3**), 60 (**M4**), 120 (**M5**) min after the drink.

L-theanine seemed to accelerate the normalization of EEG spectral power in high frequency waves (Barthel in this congress). The physiological return of increased hormon levels to basal levels / the circadianic rhythm up to **M2** (catecholamines) or **M5** (cortisole, serotonine, prolactine) was not influenced by the drinks. But in the L-theanine trials correlations between EEG spectral power and some hormones were altered (slow wave power/some catecholamines except norepinephrine/delta disappeared and new correlations with prolactine appeared). Thus we conclude that L-theanine acts at the switch from the brain to the peripheral stress regulation and thereby supports physiological relaxing after severe exercise.

Polyamines

Polyamine inhibitors and analogues interfere with the germination of kiwi pollen

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Bis(guanylhydrazones) are a class of compounds known to interfere with the metabolism of polyamines by virtue of their ability to inhibit S-adenosylmethionine decarboxylase (SAMDC), a key enzyme of polyamine biosynthesis. This property has made them useful tools to study the biological functions of these compounds. A curious feature of bis(guanylhydrazones) is their structural relationship with two molecules involved in polyamine biosynthesis, namely spermidine and S-adenosylmethionine. The methylglyoxal derivative of bis(guanylhydrazones), MGBG, has been actively studied both in animal and plant systems.

In the present work the male pollen from *Actinidia deliciosa* has been utilized to investigate the role of polyamines on the pollen tube growth. The effect of several bis(guanylhydrazones) was tested on pollen germination, length of pollen tube, levels of free and conjugated polyamines and SAMDC activity.

All bis(guanylhydrazones) tested (glyoxal-bis-guanylhydrazone, GBG, methylglyoxal- MGBG, methylpropylglyoxal-MPGBG, ethylmethylglyoxal- EMGBG) inhibit pollen germination and their effect is dose-dependent. A clear reduction of spermidine, both in free and conjugated form, was observed, as well as a pronounced decrease in SAMDC activity. These results suggest that the mechanism by which bis(guanylhydrazones) reduce the germination of kiwi pollen is related to their effect on spermidine biosynthesis.

Molecules structurally related to polyamines (1,8-diaminooctane, 1,9-diaminononane, 1,10-diaminodecane) and other inhibitors of their metabolism (cyclohexylamine, CHA) are also tested on kiwi pollen germination.

Polyamines metabolism during somatic embryogenesis in grapes

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In the frame of the study aiming to enlighten developmental programs during regeneration in grapes, polyamine content (free and conjugated to hydroxycinnamic acids) and biosynthetic enzyme activities were assayed during somatic embryogenesis. Aliphatic polyamines are growth regulators affecting plant growth and development both *in vivo* and during *in vitro* cultures, being involved in several morphogenic processes related ti their action in cell division. The study was conducted on samples of callus, embryogenic callus, embryo at different stages and plantlets of *Vitis vinifera* Brachetto and Chardonnay cultivars induced from anthers and ovaries. Polyamine content (putrescine, spermidine and spermine) free plus conjugated to percloric acid soluble fraction, referred to unit, was higher in the cv. Brachetto than in the cv. Chardonnay, and reached the higher levels in the fully-developed embryo stage. Besides, ornithine decarboxylase activity resulted higher than arginine decarboxylase and during the development from callus to plantlets, both activities increased, reaching the maximum at this latter stage. Also S-adenosylmethionine decarboxylase activity displayed a similar trend. All the activities were present in supernatant and in particulate fraction. Higher activity of enzymes assayed in the small embryos rather than in the embryo with higher shape, was consistent with following polyamine accumulation.

Incorporation of polyamines by transglutaminase into rabbit eye lens βB2/βB3-crystallins enhances their proteolytic cleavage by calpain II

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Intracellular transglutaminase (TG, EC 2.3.2.13), which catalyzes the formation of ε-(γ-glutamyl)lysine isopeptide cross-links between polypeptides, has been related to a variety of important biological processes and in the development of senile cataract. The majority of the dry weight of the eye lens is composed of protein called crystallins. In the mammalian lens, these proteins are divided into three major classes: α-, β- and γ-crystallins. Native β-crystallins are oligomers, which elute in two or more size classes during gel filtration, ranging from 200–50 kDa. They contain 7 different types of subunits, named βB1, βB2, βB3, βA1, βA2, βA3, BA4, ranging from 31–32 kDa. In the rabbit eye lens two β-crystallin subunits (βB2 and βB3), among the water soluble proteins, have been shown to act selectively as acyl donors substrates for lens TG. Calpains are cytoplamic Ca^{++}-dependent cystine proteinases. The cleavage of α- and β- crystallins, the main substrates of lens calpain II, has been associated to the increase of lens turbidity, due to insolubilization of peptides. We observed that TG-induced post-translational modification of βB2- and βB3-crystallins with polyamines, enhances their cleavage by calpain II. This finding suggests that the enhancement of calpain II activity, after conjugation of polyamines into β-crystallins, could represent an important regulatory mechanism which may contribute to the opacification process of the eye lens, conducting to cataract formation.

Functional roles of transglutaminases, a family of enzymes catalyzing crosslinkage or polyamidation of proteins

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Transglutaminases represent a family of enzymes, widely diffused in nature, from bacteria to plants and higher animals. The present discussion will focus on 4 isoenzymes in mammals, which have been well characterized from the structural and functional point of view. They act on tissular proteins catalyzing crosslinkage through isopeptide bonds at peptidyl glutamine and lysine residues or incorporation of small molecular weight primary amines, usually polyamines, in an irreversible, calcium dependent reaction. In several instances the expression of transglutaminases is regulated at the transcriptional level. These enzymes help in maintaining structural integrity of tissues intervening in wound repair and in cellular homeostasis at the levels of cell activation, receptor signaling, cell proliferation, differentiation and death. These general roles involve

multiple catalytic processes: the receptor signaling activity (demonstrated only for isoenzyme 2) is related to an intrinsic GTP-ase activity of type 2 transglutaminase; the processes leading to control of cell proliferation, differentiation and death are mainly related to the protein crosslinking activity, while the cell activation is tentatively considered dependent on the polyamidation of endogenous proteins at glutamine residues. The knowledge on this last aspect lies far back in comparison to the other roles of transglutaminases and requires further accurate investigation, which must further extend to the role of the enzyme in human pathology.

Vitamin B12 has important place in regulation of polyamine metabolism

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The examination of polyamine metabolism at the present time suggests that vitamin B12 is implicated in polyamines metabolism. Literature data speak that spermine and spermidine stimulate activity of cobalamin-dependant methionine synthase, the enzyme that catalyses the recycling of homocysteine to methionine; polyamines inhibit methionine adenosyltransferase. Beside the wellknow significance of vitamin B12, in transmethylation reaction, the significance of 5,-deoxyadenozyl cobalamin, except the conversion of methylmalonyl-CoA to succinyl CoA, is not well elucidates.

Methionine as S-adenosylmethionine (SAM) is essential amino acid for polyamine biosynthesis. SAM has frequently usage in treatment of liver diseases. According the mentioned facts the aim of our experiments is to exanimate the significance of application of vitamin B12 alone and altogether with methionine to rats without and with experimentally induced cholestasis. Our preliminary results speak about the disturbance of polyamine metabolism in hepatic tissue of rats with cholestasis. Application of methionine alone increases the amount of polyamine in rat liver tissue, in-group without cholestasis and with bile duct obstruction. The animal treatment with cobalamin has higher amount of polyamines and lower activity of polyamine oxidase in liver tissues in both groups. The effects of vitamin B12 may be in direct relation with the formation of 5,-methylthio deoxyadenosine (MTA), the by-product of spermidine and spermine biosynthesis. The explanation the exact roles of vitamin B12 in polyamine metabolism of liver tissue need the futher investigations.

Selective accumulation of putrescine in ornithine decarboxylase overproducing cells induces caspase dependent apoptosis

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Exposure of mouse myeloma cells that massively overproduces ornithine decarboxylase (ODC) but not of parental cells to ornithine results in a massive increase in the intracellular concentration of putrescine, followed by rapid cell death. The treated ODC overproducing cells display fragmented nuclei, chromatin condensation and an oligonucleosome-size DNA "ladder"; consequently, their death can be described as apoptosis. The apoptotic process induced by the accumulated putrescine involves the release of cytochrome c from the mitochondria, activation of caspases cascades demonstrated by the cleavage of caspase-2 and PARP, a substrate of caspase-3. The general inhibitor of caspases, BD-fmk, effectively inhibited PARP cleavage but failed to inhibit cell death. The intracellular Ca^{2+} chelator BAPTA/AM and the antioxidant BHA inhibit PARP cleavage. However, only BAPTA/AM inhibit the induction of cell death. It seems that BHA subverted the death into caspase independent pathway. Treatment with BAPTA/AM did not interfere with the accumulation of putresine following ornithine treatment, suggesting that the accumulated putrescine induces the elevation in the concentration of intracellular Ca^{2+} which then activates the apoptotic process. The dominant anti-apoptotic effect of BAPTA/AM over EGTA suggests that internal stores are the main source of the elevated Ca^{2+}, but that putrescine is also capable of inducing influx of extracellular Ca^{2+}.

Effects of human growth hormone treatment on amine metabolism in rats subjected to extensive small bowel resection

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Extensive small intestine resection results in the loss of absorptive surfaces, acceleration of intestinal transit and, as a consequence, in malnutrition, weight loss, diarrhoea and other complications of short bowel syndrome. The availability of human recombinant growth hormone rGH and its stimulatory effects on gut growth suggested its use in the treatment of short bowel syndrome. The trophic response of GI tract epithelium to hormones such as growth hormone is mediated by polyamines, which are vital in cell proliferation. This study was undertaken in rats to: 1/ evaluate the effects of rGH by monitoring polyamine and amine metabolism parameters in the adapting short bowel and 2/ determine whether erythrocyte (RBC) polyamine concentrations reliably reflect the proliferative activity of the remaining bowel.

Seventy per cent resection of the small intestine of Wistar rats was performed under ether anesthesia leaving equidistant lengths of bowel from pylorus and ileocecal valve. Recombinant human GH (0.2 IU, s.c., Saizen, Serono, Switzerland) was administered once daily for 5 or 10 days, to randomly selected rats on the second postoperative day. Animals were sacrificed 8, 13 and 21 days after the operation. Enzyme activities were measured with radioassays or fluorimetry. Polymines were determined as dansyl derivatives by HPLC/fluorimetry.

GH treated animals had significantly higher intestinal ODC and SAT and lowel DAO activities; higher (non-significant) mucosal growth index and polyamine concentrations than in untreated counterparts on 8th postoperative day. Thereafter the two groups did not differ in the investigated parameters. RBC polyamine concentrations were higher in operated verses control rats; rGH treatment had no significant effect. However, rGH treatment significantly reduced hepatic MAO A and B activities.

Our results suggest GH accelerated the adaptive growth of the bowel remnant. They justify use of erythrocyte polyamine concentration measurement as the marker of small bowel proliferative activity. However, side-effects of this treatment must be considered.

Tissue transglutaminase in NMDA-induced excitotoxicity

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Tissue transglutaminase (tTG) activity has been evaluated in different neural tissues, such as brain, spinal cord and peripheral ganglia, and appears to be expressed in cerebellar granule cells (CGN) as well as in astrocytes. The role of tTG in neuronal functioning is likely to be quite complex. Other than the role during development, significant changes of enzyme activity have been evaluated in different neurodegenerative conditions.

It is well known that NMDA receptor activation may be able to trigger excitotoxicity. The NMDA-induced injury is mainly associated to ion influx and subsequent calcium overload. The effects of NMDA application to both, cerebellar granule cells and glial cell cultures, have been assessed. In CGN, tTG activity increased rapidly after a brief stimulation with 100 μM NMDA, whereas in glial cell cultures, high levels of enzyme activity was obtained after incubation of 24 h in presence of the same concentration of NMDA.

Such results rule out the possibility that excitotoxicity can modify numerous proteins making them better substrates of tTG, and this could contribute to enhanced tTG-modifications of proteins in response to excitotoxicity.

Identification of the substrate recognition site on polyamine transport proteins PotE and CadB

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The PotE protein can catalyze both uptake and excretion of putrescine. The *Km* values of putrescine for uptake and excretion (putrescine-ornithine antiport) are 1.8 μM and 73 μM, respectively. Amino acid residues, Cys62, Trp201, Glu207, Trp292 and Tyr425 are strongly involved in both activities, and that Glu77, Tyr92, Cys210, Cys285, Cys286 and Glu433 are moderately involved in the activities. Mutations of Tyr78, Trp90 and Trp422 mainly affected uptake activity, indicating that these amino acids are involved in the high affinity uptake of putrescine by PotE. Mutations of Lys301 and Tyr308 mainly affected excretion activity, indicating that these amino acids are involved in the recognition of ornithine. The putrescine and ornithine recognition site on PotE was found to be located at the cytoplasmic surface and the vestibule of the pore consisting of twelve transmembrane segments.

The CadB protein has 30% sequence homology with PotE protein. CadB can catalyze both uptake and excretion of cadaverine. The *Km* values of cadaverine for uptake and excretion (cadaverine-lysine antiport) are 20 μM and 300 μM, respectively. It was found that two glutamate residues (Glu76 and Glu204) and four tyrosine residues (Tyr73, Tyr89, Tyr90 and Tyr310) are involved in the both activities. The difference of the substrate recognition site on PotE and CadB is discussed.

Polyamines conjugated to laminin and "Matrigel" by transglutaminase affect murine B16-F10 melanoma cells adhesion and invasion

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Tissue transglutaminase (tTG, E.C. 2.3.2.13) is a protein cross-linking enzyme which catalyzes an acyl transfer reaction where the carboxamide group of a peptide-bound glutamine is the acyl donor, and a lysine residue the acyl acceptor. Polyamines may act as acyl acceptors, leading to the formation of mono- and bis-(γ-glutamyl)derivatives. We provided evidence that tTG activity is directly associated to differentiation markers, and inversely related to cell proliferation and invasion. We have shown the *in vivo* reduction of experimental melanoma metastasis by i.v. injection of a plasmid (pSG5) carrying the tTG gene sequence to C57BL6/N mice.

Tumor cell metastatization requires specific interactions with subendothelial basement membrane (BM) and migration through the endothelial wall, allowing the colonization of the target tissue. Therefore, the investigation on the possible mechanisms responsible for tTG effects is focused on the post-translational modification of BM proteins. We detected that "Matrigel", a tumor-derived complex of BM proteins, modified with polyamines after tTG catalysis, reduces both melanoma cell adhesion and invasion in an *in vitro* metastatic assay. Similar results were obtained using polyamines conjugated to laminin, one of the major BM components, as unique substrate. Our findings suggest that the increase of BM proteins conjugated to polyamines may be responsible for impairments of the invasive properties of melanoma cells.

Interferon-alpha induces apoptosis in human lung cancer through tissue transglutaminase activation and hypusine reduction

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We demonstrated that Interferon-α (IFNα) induces apoptosis in human epidermoid cancer cells. Tissue transglutaminase (tTGAse) is an enzyme involved in the regulation of apoptosis through the inactivation of some cell components. Among these eukaryotic initiation factor-5A (eIF5A) is peculiar because its activity is modulated by the formation of the amino acid hypusine. Recently, we found that growth inhibition induced by tTGAse is paralleled by reduced hypusine levels.

Here we report the effects of IFNα on the apoptosis, tTGAse modulation and eIF5A activity in human epidermoid lung H1355 cancer cells. We have found that 48 h exposure to 2,500 IU/ml IFNα induces 50% growth inhibition and 15% apoptosis in H1355 cells. Moreover, IFNα induced a 4-fold increase of tTGAse activity and expression that already occurred after 24 h of exposure to the cytokine. This effect was paralleled by a 1.6-fold enhance of tTGAse mRNAs. IFNα induced also a 30% increased eIF5A expression while an about 50% decrease of hypusine levels was observed.

Increased tTGAse activity was paralleled by a decrease of hypusine content and of eIF5A activity. Therefore, IFNα-induced apoptosis could occur through an increase of tTGAse activity and the mechanism by which tTGAse regulates biological functions can be the reduction of eIF5A activity.

Polyamine depletion causes early embryonic lethality in AdoMetDC deficient mice

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The *AMD1* gene encodes *S*-adenosylmethionine decarboxylase (AdoMetDC) that is one of the key enzymes of polyamine biosynthesis. To examine the physiological role of polyamines, we performed the targeted disruption of the gene in mice to generate spermidine- and spermine-free mice. Although the level of AdoMetDC mRNA decreased by 50% in $AMD1^{+/-}$ mice, AdoMetDC activity reduced only by 30% and spermidine and spermine contents did not change significantly. They showed normal phenotype and life span. To obtain $AMD1^{-/-}$ mice, we intercrossed $AMD1^{+/-}$ mice and determined the genotype of the resulting offspring. However, we could not obtain any $AMD1^{-/-}$ mice from 20 heterozygous intercrosses over. $AMD1^{-/-}$ embryos died early in development, between E3.5 and E6.5 days post coitum. In culture of blastocysts at E3.5, the shapes of all cell lines were normal, but $AMD1^{-/-}$ cells appeared to arrest the cell proliferation at day 3 after the onset of cell culture. The arrest of $AMD1^{-/-}$ cell proliferation was rescued by addition of spermidine. These data indicated that the lethal phenotype of $AMD1^{-/-}$ mice was caused by growth retardation by polyamine depletion at early developmental stage.

New perspectives on the role of amine oxidases in physiopathology

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The formation of active species such as H_2O_2 and aldehydes during the oxidative deamination of biogenic amines by amine oxidases (AO) suggests for these enzymes a key role in cellular processes.

The ability of bovine serum amine oxidase (BSAO) to oxidase free amino groups of lysozyme and ribonuclease A has been observed indicating a possible AO involvement in the post-translational protein modification. Furthermore, BSAO inhibition by H_2O_2 formed during substrate oxidation under limited turnover conditions was demonstrated, which may be relevant to cellular physiopathology. We have also observed that some inhibitors of mitochondrial amine oxidases (MAO) protected human melanoma cell line (M14) against apoptosis. The protection by catalase of MAO-substrates induced membrane permeability transition was also obtained in isolated rat liver mitochondria, thus confirming a role of MAO-derived H_2O_2 in apoptosis. Enrichment in AO activity by treatment with vegetal AO has been obtained in a erythroleukemia cell line (K562), substaining the possibility to modulate the intracellular AO activity. An antiarrhythmic and cardioprotective effect of BSAO has been also observed on isolated rat heart in reperfusion; a protective effect during anaphylaptic crisis has been shown "*in vivo*", thus suggesting AOs as a possible therapeutic agents.

Tetrakis(3-aminopropyl)ammonium, a unique polyamine produced by an extreme thermophile, stabilizes nucleic acids at high temperature

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An extreme thermophile, *Thermus thermophilus*, produces tetrakis(3-aminopropyl)ammonium; a novel polyamine containing a quaternary ammonium nitrogen. To clarify the roles of the unique polyamine in thermophily, the effects of tetrakis(3-aminopropyl)ammonium on biochemical reactions related to nucleic acids have been investigated. The unique polyamine stabilized both double and single stranded DNAs and RNAs. Tm of a double stranded DNA was raised by 8°C by the addition of 0.25 mM of tetrakis(3-aminopropyl)ammonium. At around the boiling temperature of water, depurination of DNA takes place. Other long polyamines produced by the thermophile such as caldopentaamine also stabilized DNAs and RNAs. We found that tetrakis(3-aminopropyl)ammonium prevents depurination most effectively. Tetrakis(3-aminopropyl)ammonium activated the protein biosynthesis catalyzed by a cell-free extract of the thermophile at high temperature. The effects of this unique polyamine on DNA and RNA polymerases are also being investigated and the results will be presented.

Identification of tissue transglutaminase-reactive residues in glyceraldehyde 3-phosphate dehydrogenase using as probes polyamines and peptides

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Tissue transglutaminase (tTG) catalyses the cross-link formation between glutamine (Q) residues and NH_2-donor molecules present in the cells (polyamines, lysine-donor proteins). Recently, it has been correlated to neurodegenerative disorders characterised by polyglutamine (Q_n) expansion, like Huntington's disease. Studies carried out on cell extracts revealed that glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was found covalently linked to Q_n domains. However, to date no structural data are available to solve the issue of which residues of GAPDH are substrates for tTG. By coupling classical protein chemistry procedures and mass spectrometric techniques we achieved this goal by using as tTG substrates the Substance P, an 11-aa peptide bearing the simplest Q_n domain (Q_2), and polyamines of different size and shape as Q- and NH_2-donor, respectively. In the present study we report that out of the 26 lysines present in GAPDH only three are sites of tTGase-dependent cross-link formation in vitro. Moreover, to characterize the tTG catalysed cross-link between GAPDH and polyQ protein we used a synthetic Q_{17}-peptide as tTG substrate in the catalysed reaction with polyamines. We found that any Q residue is a potential tTG substrate, no matter the specific position in the sequence or the steric hindrance of the specific amine under investigation.

Attempts to inhibit polyamine metabolism for cancer therapy

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As soon as the key role of ODC in polyamine metabolism was recognised, it became the major target for selective inhibi-

tion. S. Harik presented in 1973 the first potent ODC inhibitor, α-hydrazino ornithine. Although efforts continued until today, with the aim to improve ODC inactivation, 2-(difluoromethyl)ornithine (DFMO) remained the most important compound among all polyamine-directed drugs. A known anti-leukaemic drug, methylglyoxal-bis(guanylhdrazone), was recognised early on by G. Williams-Ashman and his collaborators as an inhibitor of AdoMetDC, the other highly regulated biosynthetic decarboxylase, and served as matrix for more recent developments. In the course of the years selective inhibitors for all enzymes involved in polyamine biosynthesis and degradation were synthesised. Moreover, a series of polyamine-uptake inhibitors were reported. However, only some of these numerous compounds reached a stage above evaluation as growth inhibitors of cancer cells. Owing to the sophisticated homeostatic regulation of the polyamines in cells and organs by de novo synthesis, degradation, uptake and release, and due to the fact that exogenous polyamines (i.e. gut polyamines) can be utilised by the vertebrate organism, the efficacy of selective enzyme and uptake inhibitors remained modest in cancer therapy. The fact the DFMO became the most important drug for the therapy of West and Central African sleeping sickness relies on differences of vertebrate and parasite biochemistry. A novel approach, initiated by Carl Porter, involved the design and synthesis of structural analogues of spermidine and spermine, which do not share the growth-promoting effects of the natural polyamines. A very large variety of homologues, mostly of spermine, with different alkyl-substituents on the primary amino groups, have been studied systematically with regard to their ability to alter enzyme and polyamine patterns, and to inhibit cell growth. In addition polymine-like chains with interposed heteroatoms (0, S, Si etc.), and analogues with rigid aliphatic chains (due to inbuilt double and triple bonds, or of small rings) have been explored. The structural analogues either mimic regulatory functions of the natural polyamines, and thus lead to the depletion of endogenous pools of putrescine, spermidine and of spermine, or they prevent growth effects of the natural polyamines by displacing them from functionally important binding sites. The later type may be considered as polyamine antagonists. The actual drugs usually exhibit to some extent polyamine mimetic and antagonist properties. At present several polyamine analogues are in clinical trial. However, after more than 25 years of active research, a polyamine-related anticancer drug is still not available. One may conclude from this fact that the polyamines are an inappropriate target for cancer treatment. However, it is more likely that polyamine metabolism is a difficult target, because the differences between normal and cancer cells are mainly of quantitative nature. Moreover, numerous mechanisms have developed in the course of evolution, which enable the vertebrate organism to prevent lethal polyamine losses.

Bis(uracilyl) polyamines analogs as potential anti tumor agents

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Nine novel chemically modified polyamine (PA) analogs were evaluated for their ability to inhibit the PA biosynthesis in rat hepatoma G-27 cell-free system as well as the growth of CaOV tumor cells. The final concentration of oxy- and amino-adenosine PA analogs or two uracils modified PA analogs were 0.1 mM in the reaction mixture. Bis(uracilyl)-analogs and 8-(2-oxyethyl)ami-no-9-β-D-xylofuranosyladenine supressed PA and putrescine synthesis and in the same conditions were more effective than DL-α-difluoromethylornithine (DFMO) – strong specific inhibitor of ornithine decarboxylase (ODC). The other adenosine modified compounds could act both as activators of ODC and inhibitors both diamine and polyamine oxidase activities in regenerating liver test system. In contrast to those mentioned above two uracils modified agents as well as DFMO were able to inhibit ODC and to increase the rate of oxidative deamination of PA in the same system. Thus bis(uracilyl) PA analogs were the most active and may be useful for further investigation as substances having potential antitumor and antiproliferative properties.

Age-related salivary polyamine increase in subjects wearing orthodontic Ni-Ti archwires

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Several studies concerning the periodontal status in adult and adolescent patients treated with fixed Ni-Ti archwires have been performed, but until now it is not yet available any information about the influence of patient age on gingival tissue responses to Ni-Ti alloy. Recently, researches by us demonstrated that the prolonged use for over 12 months of Ni-Ti appliances may contribute to local pathological proliferative processes early detectable only through salivary polyamine concentration increase. Although other data from our laboratory showed that salivary polyamine amounts are age and sex-independent, nothing is known about the influence of the age on salivary polyamine content m subjects wearing Ni-Ti appliances.

Eighty patients, under orthodontic treatment for 12 months, were divided into four groups: the pre-, the mid-, the late- and the post-pubertal. Salivary polyamine concentrations were determined by HPLC. Only the late pubertal group revealed a significant increase in both the spermine and spermidine content, while the other groups showed no modification.

The results suggest that gingival pathological responses to a long-term appliance's use may be related to the endocrine modifications that occur in the late-pubertal age.

Sexual hormones appear to be in synergy with Ni-Ti alloy in promoting proliferative activity of gingival cells.

Polyamine enhancement of the synthesis of adenylate cyclase at the translational level and the consequential stimulation of the synthesis of the RNA polymerase σ^{28} subunit

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The effects of polyamines on the synthesis of various σ subunits of RNA polymerase were studied to determine how polyamines influence the functional specificity of transcription using Western blot analysis. Synthesis of σ^{28} was stimulated 4.0-fold and that of σ^{38} was stimulated 2.3-fold by polyamines, whereas synthesis of other σ subunits was not influenced by polyamines. Stimulation of σ^{28} synthesis by polyamines occurred at the level of transcription. Since our hypothesis is that polyamines regulate macromolecular synthesis mainly at the translational level, we searched for a target protein, related to the polyamine stimulation of σ^{28} synthesis, whose translation is altered by polyamines. Stimulation of σ^{28} synthesis was due to

an increase in the level of cAMP, which occurred through polyamine stimulation of the synthesis of adenylate cyclase at the level of translation. Polyamines were found to increase the translation of adenylate cyclase mRNA by facilitating the UUG codon-dependent initiation. Analysis of RNA secondary structure suggests that exposure of the Shine-Dalgarno (SD) sequence of mRNA is a prerequisite for polyamine stimulation of the UUG codon-dependent initiation.

Synthesis

Synthesis, characterization and antitumor activity of 1,2-benzo-8-(α-hydroxy, β-aminopropyl)-3-hydroxyphenoxazine (BHMHP)

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By reacting tyrosine with 1-nitroso-2-naphthol in the presence of nitric acid 1–2-benzyo-8-(alanyl)-3-phenoxazone (BLP) an analog of actinomycin D is produced. The structural similarity of BLP to actinomycin D prompted the National Cancer Institute (NCI) to investigate its antitumor activities. The NCI investigations revealed that BLP exhibits growth inhibitory effects on various cancer cells and as a result BLP has received the U.S. Patent from the U.S. Patent Office. The purposed of this investigation was to synthesize similar benzo phenoxazone derivative by reacting 1-nitroso-2-naphthol with 4-(α-hydroxy β-methylaminopropyl)phenol in the presence of nitric acid.

During the study, it was found out that 1,2-benzo-3-phenoxazone derivative is not produced but a hydrogenated form of 1,2-benzo-3-phenoxazone which is probably 1,2-benzo-8-(α-hydroxy β-methylaminopropyl)-3-hydroxyphenoxazine (BHMHP) which has been suhhested from mass spectra obtained by electron ionization, EI, chemical ionization, CI and electro-spray ionization, ESI, methods.[1]

BHMHP was screened against various cancer cell lines by NCI and has shown promising effect against three (3) breast cancer cell lines: MDA-MB-435, MDA-N and HS-578 T. The 50% growth inhibitory (GI_{50}) concentrations for these three cell lines were 4.60×10^{-6}, 4.62×10^{-6} and 5.74×10^{-6} molar respectively.

Oxy- and sulfoguanidino analogues of histamine Synthesis and biological activity

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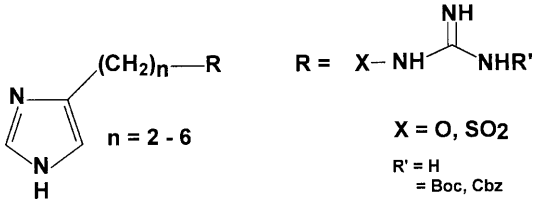
The histamine is an endogenous substance with neurotransmitter and neuromodulator functions in the organism. Its antagonists are used in the therapy of allergic diseases and inflammatory reactions and as antiulcer drugs.

The limited potentialities of the antihistamine therapy together with the increasing number of the people suffering from allergic diseases give rise to the design and synthesis of new histamine analogues as a perspective area in the chemistry of therapeutic drugs.

Additionally, compounds containing the guanidine, oxyamino and sufonamide moieties are known to elicit a variety of pharmacological responses and are present in several marketing drugs or drug candidates.

On the other hand, similar compounds, being a part of bigger structures (for instance peptides), can imitate the molecules of already known AT II-receptor antagonists.

Having in mind these data we aimed to synthesize new analogs of histamine containing sulfo- and oxy-guanidino groups with common formula:



Melanocyte-inhibiting factor (MIF-1) and its new analogues: Effects on nociception

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The problem of the efficient therapy of pain is important not only from clinical but from social and economic point of view. The great achievements in medicine are connected with the research on the development of antinociceptive drugs.

Melanocyte-inhibiting factor (MIF) is a tripeptide (Pro-Leu-Gly-NH_2) that was discovered in hypotalamus.

The MIF-1 exerted a weak analgesic effect. The synthesis of non-protein amino acids and their incorporation into biologically active peptides might become a powerful method for the design and development of modified analogues of natural peptides. Having in mind these data we synthezied a number of new MIF-analogues, containing unnatural amino acids such as Cav, sLys, sLeu, slle and sNIe and *in vivo* experiments were performed to study their action on the nociception. The changes in nociceptive effects were examined in male Wistar rats by the tail-flick (TF) and hot-plate (HP), as well as, the Randall-Seitto paw-pressure tests. The peptides were applied intaperitoneal (i.p) injection at a does 1 mg/kg. The results show that the newly sinthesized analogues exert an antinociceptive effects in all tests used. Naloxone at a dose 1 mg/kg (i.p) antagonized the antinociceptive effects of MIF-analogues.

Novel ω-N-quinonyl amino acids: Synthesis, electrochemical and spectral properties

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Quinonic compounds are ubiquitous in nature. They are implicated in numerous cellular functions and are involved in mechanisms of electron and hydrogen transfers. Many

antitumor quinones are approved for clinical use and others antitumor quinones are in different stages of clinical and preclinical development. The efficiency of the quinonic compounds in inhibiting cancer cells growth is believed to stem from their participation in key cellular redox mechanisms with consequent generation of highly reactive oxygen species (ROS). The ROS is turn modify and degrade nucleic acids and proteins within the cells. Recently, quinonic drugs were attached to the neurodecapeptide LH-RH and evaluated as potential drugs in the treatment of different tumours.

We have synthesized several series of *N*-quinonyl amino acids in which five ω-amino acids are attached to *p*-quinones with different values of redox potentials. The attachment was made *via* Michael-like reductive addition of the amino acids to the quinonic ring or *via* substitution of a chlorinated atom.

The N-ω-quinonyl amino acids were characterized as to their ability to form semiquinone anion radicals by EPR and cyclic voltammetry technique. The preparative methods, the redox potentials as well as the physical and spectral data (^{1}H-NMR, IR, UV-Vis and HRMS) of these N-ω-quinonyl amino acids will be presented.

Design of novel amino acids for examination of peptide and protein structure-biological relationships in chi space

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The *de novo* design of biologically active peptides and proteins, mostly has involved consideration and design of backbone conformations (secondary structures) such as α-helix, β-sheets, β-turns, etc. (η/ψ space). However, for many bioactive peptides and proteins, especially those critical for information transduction such as neurotransmitters, hormones, antigens, neurocrines, etc. molecular recognition via side chain moieties is of paramount importance. Thus far, the specific three dimensional orientations of side chain groups (χ angles; chi space) in terms of biological activity has received only modest attention. In part this may be due to the energetics of chi space compared to Ramachandran space. In order to overcome the current limitations of evaluating the importance of chi space in critical biological functions related to disease and behavior, we have designed amino acids with novel structures and unique constraints in chi space (χ^1, χ^2, etc.), with special attention to their ability to mimic the chi space of native proteins and peptides. We have developed novel and simple asymmetric synthetic methods for such amino acids, often with ees greater than 98%. Incorporation of these novel amino acids into bioactive polypeptide neurotransmitters has provided ligands with unique biological activities that effect unique behaviors including feeding, sexual, pain, and addictive behaviors.

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Quantitation and facilitated de novo sequencing of proteins by isotopic N-terminal labelling of peptides with a fragmentation-directing moiety

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We describe a method for comparative quantitation and de novo peptide sequencing of proteins separated either by standard chromatographic methods or by one and two-dimensional polyacrylamide gel electrophoresis. The approach is based on the use of an isotopically labelled reagent to quantitate (by mass spectrometry) the ratio of peptides from digests of a protein being expressed under different conditions. The method allows quantitation of the changes occurring in spots or bands that contain more than one protein, and has a greater dynamic range than most staining methods. Since the reagent carries a fixed positive charge under acidic conditions and labels only the N-terminal of peptides, the interpretation of tandem mass spectra to obtain sequence information is greatly simplified. The sequences can easily be extracted for homology searches instead of using indirect mass spectral based searches and are independent of post-translational modifications.

A new method for the synthesis of (E)-α,β-didehydro-α-amino acid derivatives: scope and limitations

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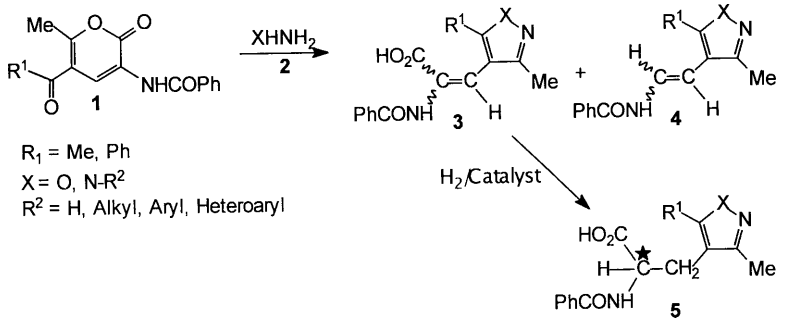
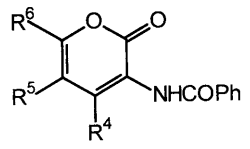
Dehydroamino acids and their derivatives play important roles as constituents of various natural products and as synthetic intermediates for the preparation of optically pure amino acids. A large number of amino acid derivatives containing a pyrazol-4-yl, isoxazol-4-yl and other heterocyclic moieties has been prepared as potential agonists or antagonists for central glutamate receptors in connection with (*R*,*S*)-2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propanoic acid (AMPA), a bioisostere of (*S*)-glutamic acid. β-Hetaryl-α,β-didehydroalanines might be considered as conformationally constrained AMPA analogs and might be potential candidates for the synthesis of novel types of AMPA analogs, for example, via their hydrogenation. Compounds containing 2*H*-pyran-2-one ring are also very useful synthons in selective synthesis. Recently we have shown their use for the preparation of (*E*)-α,β-didehydro-α-amino acid derivatives containing a pyrazolyl moiety (Vraničar L, Polanc S, Kočevar M (1999) Tetrahedron 55: 271).

As a continuation of our investigation in this field we report here a detailed study of the transformation of 2*H*-pyran-2-one derivatives **1** with hydroxylamine (**2**, X = O) and various hydrazines (**2**, X = NR^2) towards novel types of (*E*)- and (*Z*)-α,β-didehydroamino acid derivatives **3**. In most cases, the reactions were performed under basic conditions in a mixture of





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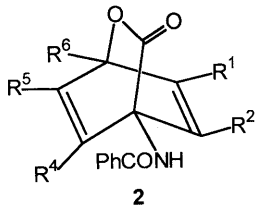

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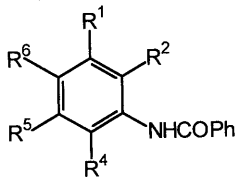

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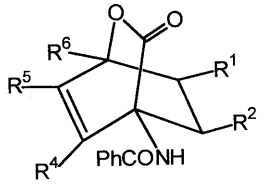

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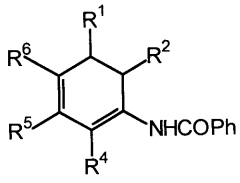

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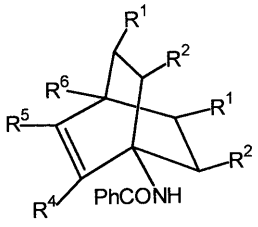

6

ethanol and pyridine. Depending on the substrate and the reagent used the reaction could be controlled to give either (*E*)- or (*Z*)-isomers; in some cases decarboxylation to the corresponding enamines **4** also occured during the reaction course. Some attempts to hydrogenate compounds **3** towards α-amino acid derivaties 5 by homogeneous or heterogeneous catalysis were also performed.

Opioid analog peptide alcohols

L. Kocsis[1], **I. Lengyel**[2], **A. Borsodi**[2], **A. Z. Rónai**[3], **M. Al-Kharasani**[3], and **G. Orosz**[4]

[1]Department of Organic Chemistry, Eötvös University, [2]Biological Research Center of Hungarian Academy of Sciences, Szeged, [3]Semmelweis Medical University, Budapest, and [4]Research Group of Peptide Chemistry, Hungarian Academy of Sciences, Budapest, Hungary

Analogs of endomorphin 1 and 2 were prepared to investigate the effect of the positional and C-terminal amide replacements and modifications on the biological activity. Modifications in position 2 and 4 were studied. In position 2 several hydroxy- and serine related amino acids were incorporated, whereas in position 4 the amide bond was replaced by hydroxymethyl and allyl group.

Protected peptide derivatives were synthesized on 2-chlorotrityl resin and further transformed to the corresponding derivatives in solution phase.

Among the analogs tested, in *in vitro* tests the most effective compound found was D-Ser[2]-endomorphin −2.

Quite surprisingly, the partial agonist/antagonist properties of the derivatives in receptor binding and G-protein stimulation tests have been shown behave differently. The differences in efficacy and receptor binding properties of the compounds may explain the discrepancies between the in vitro and receptor binding tests.

Cycloadditions with amino acid derivatives containing *2H*-pyran-2-one moiety: high pressure versus thermal reactions

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Faculty of Chemistry and Chemical Technology, University of Ljubljana, Slovenia

We have been assessing the possible applications of substituted 2*H*-pyran-2-ones **1** containing α,β-didehydroamino acid unit in their structure as dienes in [4+2]-cycloaddition reactions. As dienophiles we have been using different acetylene derivatives as well as *N*-phenylmaleimide and maleic anhydride. As it is evident from the structure of 2*H*-pyran-2-ones upon the cycloaddition of acetylene derivatives the first intermediate formed (**2**) still contains the carbon dioxide bridge. In many cases **2** easily expels CO_2 and substituted benzene derivative **3** is produced. When the alkenes are used, the first part of cycloaddition is the same as when acteylene derivatives are used, but after the extrusion of CO_2 from the adduct **4** there are two possible paths: so formed cyclohexa-1,3-diene (**5**) is either aromatized into benzene derivative (**3**) or it acts as another diene with favourably positioned double bonds and unusual double cycloadducts (**6**) are formed. Since CO_2-containing adducts are thermally unstable it is advantageous to use high pressure techniques. With the acetylene derivatives we have not been able to isolate CO_2-containing adducts (**2**), while with alkenes we have isolated, depending on the structure pattern of the compound **1**, all three types of products: aromatized **3**, CO_2-containing **4** and double adducts **6**. Especially the type **4** is suitable for further transformations into other heterocycles containing amino acid moiety.

Synthesis of nociceptin analogues containing carbamoyl bond instead of peptide bond

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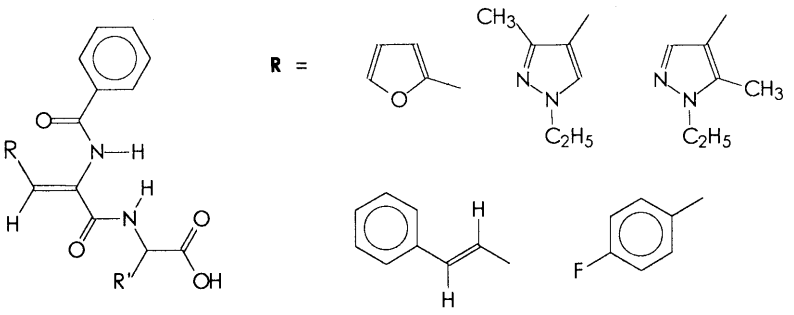
Research Group of Peptide Chemistry, Hungarian Academy of Sciences, Budapest, Hungary

Among the opioid receptors family, the cloning of μ, κ and δ receptors was followed by another member, named LC_{132} or ORL_1. Searching for an endogenous ligand for this receptor resulted in successful identification of a peptide (FGGFT GARKSARKLANQ) called Noc or OFQ. In vitro and in vivo studies have demonstrated that Noc mediates a variety of biological actions.

Results from structure-activity experiments suggest that the whole sequence of Noc is not required for binding to the LC_{132} receptor and for full biological activities. Noc(1–13)-OH seem to be the minimum and essential sequence for good interaction with the receptor.

This neuropeptide, similarly other peptides, are unresisting for enzymatic degradation and the releasing metabolites are very weakly active or inactive. Some previous experiments refer to that the C-terminal amidation may protect the peptide from degradation. We purposed to synthesize carbamoyl



analogues of Noc(1–13)-NH_2, hoping that these derivatives retain the ability to bind LC_{132} receptor and are resistant against biological degradation:

Phe-NH-CO-β-Ala-Noc(4-13)-NH_2
Phe-β-Ala-NH-CO-Phe-Noc(5-13)-NH_2
Phe-Gly-NH-CO-β-Hphe-Noc(5-13)-NH_2

The first step in the synthesis of the carbamoyl analogues was the preparation of the building block [R-CO-NH-CO-NH-HC(R′)-COOH] by the classical method and then it was incorporated into the peptide by solid phase peptide synthesis.

[This work was supported by grand from the Hungarian Research Foundation (OTKA 033078).]

Ca and Mg complexes for the asymmetric synthesis of dipeptides containing nonproteinogenic amino acids

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A. N. Nesmeyanov Institute of Organo Element Compounds, Russian Academy of Sciences, Moscow, Russian Federation

Nonproteinogenic amino acids and their derivatives are valuable compounds from their pharmacological and biochemical effects. They can be used also in synthesis of peptides, as biomarkers, as the ligands in catalitically active transition metal complexes and so on.

It is possible to prepare such amino acids by asymmetric hydrogenation of their prochiral precursors. However high enantioselektivities was reached only in the case of chiral phosphine-rhodium catalysts.

Recently we showed that high diastereoselectivity in the hydrogenation of linear dehydrodipeptides may be achieved over achiral catalyst in the catalytic system substrate – salts of Ca ore Mg – Pd/C due to formation of dehydrodipeptides complexes with ions $Ñà^{2+}$ or Mg^{2+} and hence increasing of the conformational rigidity of substrates. This phenomenon may as well happen in other dehydrodipeptides, containing nonproteinogenic amino acids. Among unnatural amino acids those bearing heterocyclic rings have attracted considerable attention due to the possibility of the heteroatoms participation in coordination with ions of metals.

We have received some N-acyldehydrodipeptides, containing in the prochiral unit of dipeptides nonproteinogenic dehydroamino acids.

All this N-acyldehydrodipeptides form in alcohol solution complexes with CaX_2 and MgX_2 where one metal ion binds together several (up to 5) substrate molecules. This kind of complexation leads to the increase of conformational rigidity and to the diastereoface shielding of C=C bond. Moreover the combination of cations (Ca^{2+} or Mg^{2+}) and anions (X) and the sequence of their mixing with a substrate determine the assembly inside complex particles and hence the sign and degree of asymmetric induction.

Indeed hydrogenation of these complexes formed in situ over achiral heterogeneous catalyst (Pd/C) gives two diastereomers of corresponding N-acyldipeptides with the substantial increase of the reaction diastereoselectivity (up to 80%).

Synthesis and biological evaluation of optically active fluorinated analogues of glutamine

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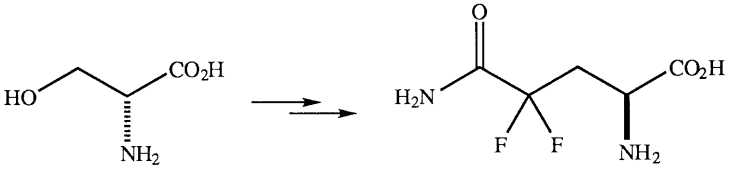
In living cells, glutamine represents one of the main storage forms of nitrogen and is a major physiological source of ammonia for the biosynthesis of aminoacids, aminosugars, purine and pyrimidine nucleotides and coenzymes.

Glutamine-dependent amidotransferases perform nitrogen transfer from the amide group of glutamine to various electrophiles. When the latter is fructose-6P, the product of the reaction catalysed by Glucosamine-6P synthase is D-glucosamine 6-phosphate, a structural building block of peptidoglycane (bacteria) and of chitin and mannoproteins (fungi).

Fluorinated analogues of glutamine are expected to interfere with this biological process due to the strong electron withdrawing effect of fluorine atom (without significant steric consequence), inducing modulation of binding and/or electronic properties. These compounds might therefore behave as reversible or irreversible active site-directed enzyme inhibitors.

Synthesis of optically active **1** from D-serine will be described and first results in the biological evaluation on Glucosamine 6-phosphate synthase will be included.



D-serine L-4,4-difluoroglutamine **1**

Chemoselective synthesis of lipopeptides. Application to the synthesis of novel interferon-gamma agonists

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1-UMR 8525, 2-Sedac-Therapeutics, Biological Institute of Lille, France

Lipopeptides, owing to their ability to cross passively the cell membrane or biological barriers, are unique tools for the intracellular delivery of bioactive peptides. The structure of the lipophilic moiety is known to have a profound effect upon the interaction with the membrane and its alteration. The stepwise solid phase synthesis of lipopeptides is limited by the necessity to perform a complex RP-HPLC purification following the cleavage and deprotection step. In addition, the harsh conditions used during the final acidolysis procedure does not allow the introduction of unsaturated or sensitive fatty acids. To speed up the access to large lipopeptides modified by various fatty acid moieties or cholesterol derivatives, we have designed novel synthetic methods which involve the chemoselective reaction of fully deprotected and purified hydrazinopeptides with fatty acid succinimidyl esters or glyoxylyl derivatives. Application of these methodologies to the C-terminal 95–135 portion of interferon (IFN)-γ allowed the selection of the optimal lipopeptide IFN-γ agonist, as determined by its ability to induce the expression of surface MHC-II molecules through interaction with the intracellular components of IFN-γ receptor.

Asymmetric synthesis of α-substituted glutamate analogs

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Graduate School of Science, Osaka City University, Osaka, Japan

Glutamate receptors in mammalian CNS are implicated in the construction of memory and early learning as well as in the pathogenesis of neuron damage to cause various neuronal diseases. In recent years, we have studied the conformational role of L-glutamate when it binds to the receptors through the synthesis of L-2-(carboxycyclopropyl)glycines (CCGs) and their related analogs. The works have demonstrated that not only the receptors require a specific conformation of L-glutamate, but also these analogs can be used as important tools for the neuropharmacological research. Among them, DCG-IV, a 3′-substituted analog of CCG-I, is used as a potent and selective agonist of mGluR2.

As an extension of these works, next program was focused on the synthesis of α-substituted glutamate analogs which would enable to develop potent and subtype-selective ligands for mGluRs and transporters. α-Alkoxymethylglutamate and LY354740 and its C5 epimer were chosen for the synthetic targets, since the former slightly restricts the glutamate conformation to an extended form and the latter rigidly fix to an extended or a folded form on its bicyclo[3,1,0]hexane skeleton. The key to the synthesis was a stereoselective construction of the quarternary carbon center, which was efficiently performed based on an asymmetric version of the Strecker synthesis. Details of the synthesis and their neuropharmacological activities will be described.

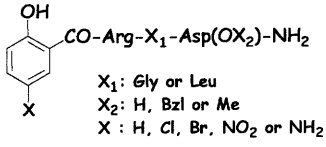
Synthesis of RGD analogs incorporating salicylic acid derivatives and their biological activity on human platelet aggregation *in vitro*

Y. Sarigiannis[1], **G. Stavropoulos**[1], **M. Liakopoulou-Kyriakides**[2], and **P. E. Makris**[3]

[1] Department of Chemistry, University of Patras,
[2] Department of Chemical Engineering, and
[3] Department of Medicine, Haemostasis and Thrombosis Unit, Aristotle University of Thessaloniki, Greece

The interaction between platelets and fibrinogen is known to be mediated by the intergrin GP IIb/IIIa. The Arg-Gly-Asp (RGD) sequence located on fibrinogen and other proteins of blood and extracellular matrix is the minimum requirement for cell attachment and adhesion. It has been found that peptides containing the RGD sequence can effectively inhibit the binding of fibrinogen to GP IIb/IIIa. In addition aspirin has been shown to be beneficial in the treatment of stable and unstable angina, acute myocardial infraction. Aspirin acetylates and inhibits the enzyme cyclooxygenase, the first enzyme involved in thromboxane A_2 (TXA_2) synthesis, an activator of platelet aggregation and adhesion.

We have already reported that the combination in the same molecule of dipeptide amides, containing amino acid(s) of RGD sequence, with salicylic-residue 2-RO-C_6H_4-CO∼, {where R = H or CH_3CO} at their N-terminal amino group have shown inhibitory activity on human platelet aggregation. Continuing this research project on salicyl-peptides we have synthesized a series of RGD analogs, incorporating salicylic acid derivatives, by conventional solution techniques and/or by solid phase. The synthesized RGD analogs were identified by IR, NMR and ES-MS spectra and tested for inhibitory activity on human platelet aggregation *in vitro*, by adding common aggregation reagents (collagen, ADP, thrombin) to citrated platelet rich plasma (PRP). Platelets were obtained from venous blood of healthy donors and the PRP was isolated by centrifugation at 200 g for 5 min at 37°C. The aggregation was determined using a dual channel electronic aggregometer. Malonyl dialdehyde (MDA) production was measured using thiobarbituric acid reagent. In order to confirm these results, flow cytometry with monoclonal antibodies against GpIb, GpIIb/IIIa, GpIIIa and GMP140 was used. The IC_{50} values of the synthesized and tested compounds, as well as their MDA production and flow cytometry results will be discussed.



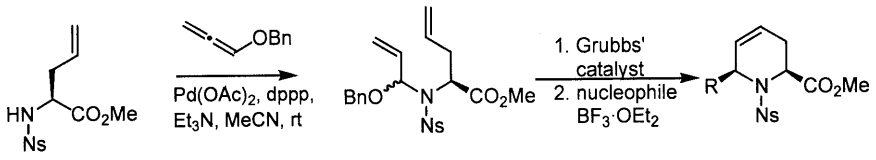
[Acknowledgment: The Research Committee of the University of Patras supports this project (Grant No 2468).]

Synthesis of enantiopure unusual amino acids through a combination of bio- and transition metal catalysis

H. E. Schoemaker

DSM Research, Life Science Products, Geleen, The Netherlands

Amino acids have a long tradition as building blocks, chiral auxiliaries and/or ligands in advanced organic synthesis and catalysis. At DSM an enzymatic kinetic resolution process has been developed, based on an aminopeptidase catalyzed stereoselective hydrolysis of racemic amino acid amides to form a mixture of L-amino acid and unchanged D-amino acid amide.



Using a genetically modified organism a broad variety of linear unsaturated amino acids are now accessible in enantiomerically pure form via this methodology, which can be used as starting materials for the synthesis of highly functionalized pipecolic acid derivatives. These compounds can be used to restrict conformations in polypeptides or can serve as scaffolds in synthesizing libraries for drug discovery. The synthetic approach involved both a Pd-catalyzed amidopalladation reaction of alkoxy-allenes, in which the NH is added across one allene double bond and a ruthenium catalyzed ring closing metathesis step, to form a benzyloxypipecolic acid. Further reaction of this N-sulfonyl-iminium-ion precursor with a nucleophile results in the formation of *cis*-substituted pipecolic acids.

Protease-catalyzed synthesis of α-fluoroalkyl substituted peptides

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Institute of Organic Chemistry, University of Leipzig, Germany

Due to the unique electronic properties of fluorine, incorporation of α-fluoroalkylated amino acids is a new approach to design biologically active peptides with increased metabolic stability and defined secondary structure and provides a powerful NMR label for spectroscopic investigations.

The application of proteases especially for CN-ligations is an attractive alternative to chemical methods, because the enzymatic formation of peptide bonds is highly regio- and stereospecific and, therefore, does not require large efforts to protect side chains of trifunctional amino acids. Recently, the enzyme-catalyzed incorporation of α-fluoromethyl amino acids into the P_2, P_3 and P_2'-position (nomenclature according to Schechter and Berger) of peptide fragments has been successfully performed. Carboxypeptidase Y was now shown to be suitable to catalyze the incorporation of α-trifluoromethyl alanine into the P_1 position of peptides. Furthermore, the general applicability of the substrate mimetic concept in enzymatic peptide synthesis was expanded to the transfer of C-terminal α-fluoroalkyl substituted amino acids. Generally, each trifluoromethyl- and difluoromethyl amino acid 4-guanidinophenyl esters can be applied as acyl donor in trypsin and chymotrypsin catalyzed peptide bond formation independently of the acyl moiety and the natural enzyme specificity, respectively. Via these two approaches, incorporation of α-fluoroalkylated amino acids into the P_1 position of peptides using enzymatic methods was successfully applied for the first time.

Synthesis of substance P C-terminal analogs incorporated D-Trp and studies of their antineoplastic properties *in vitro*

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[1]Department of Chemistry, University of Patras, [2]Department of Chemical Engineering, and [3]Department of Medicine, Aristotle University of Thessaloniki, Greece

Several small peptides currently are under investigation as possible anti-tumor agents. Neuropeptides such as substance P (SP) and neuropeptide Y (NPY), have been studied for their ability to prevent tumor growth or the proliferation of several cancer cell lines. These neuropeptides have been investigated for their effect to prostate cancer, small cell lung cancer (SCLC) and breast cancer. The synthetic SP analog [D-Arg[1], D-Phe[5], D-Trp[7,9], Leu[11]]SP (antagonist D) and the C-terminal analog [Arg[6], D-Trp[7,9], MePhe[8]]SP_{6-11} (antagonist G) inhibit SCLC cell proliferation in vitro and in vivo, while the analogs [Glp[6], Glu(Bu^t)[11]]SP_{6-11} and [Glp[5], Glu(Bu^t)[11]]SP_{5-11} showed significant inhibition in the proliferation of the cancer cell lines HeLa and T47D.

In the present study the C-terminal analogs of SP [Glp[6], D-Trp[7], Glu(Bu^t)[11]]SP_{6-11} (**1**), [Glp[6], D-Trp[7,9], Glu(Bu^t)[11]]SP_{6-11} (**2**), [Glp[6], D-Trp[7,9], MePhe[8], Glu(Bu^t)[11]]SP_{6-11} (**3**), [Glp[6], D-Trp[7], MePhe[8], Glu(Bu^t)[11]]SP_{6-11} (**4**), [Glp[6], Trp[7], MePhe[8], Glu(Bu^t)[11]]SP_{6-11} (**5**), [Glp[6], MePhe[7], D-Trp[8], Glu(Bu^t)[11]]SP_{6-11} (**6**), [Glp[6], D-Trp[7], MePhe[8], Glu(Bu^t)[11]-OH]SP_{6-11} (**7**), [Glp[6], D-Trp[7], Cys(Acm)[11]-OH]SP_{6-11} (**8**), [Glp[6], D-Trp[7], MePhe[8], Cys(Acm)[11]-OH]SP_{6-11} (**9**), [Glp[6], D-Trp[7,9], MePhe[8], Cys(Acm)[11]-OH]SP_{6-11} (**10**) have been synthesized and tested for their antineoplastic properties in several cancer cell lines. They were also examined for their cytotoxicity to normal cells.

The analogs **1–6** are peptide amides whereas the analogs **7–10** are peptide acids. They were performed using the stepwise synthesis either in solution, using the method of mixed anhydrides with carbonic acids or in SPPS using the Fmoc/Bu^t methodology. The fragment condensation method in solution, using phosphonium reagents, such as PyBOP, was also applied. The analogs were purified (HPLC) and identified (FT-IR, ES-MS, ^{1}H-NMR).

The antineoplastic properties of the analogs were studied using Sister Chromatide Exchange (SCE) and Proliferation Rate Index (PRI). As it is known the SCE method is an indicator of DNA damages or its repair mechanism, while the method of PRI is a sensitive marker of cytotoxicity. The experiments were carried out using cultured human lymphocytes from healthy donors and these results will be discussed.

Semiempirical quantum chemical investigation of some thymidine derivatives modified with amino acids and peptides at 3′, 5′-positions

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[1]Department of Chemistry, South-West University "Neophit Rilski", Blagoevgrad, and
[2]Department of Chemistry, Sofia University "St. Kl. Ohridsky", Sofia, Bulgaria

Optimized geometry and electron charge distribution for some thymidine derivatives (3′,5′-bis-O-N-α-benzyloxycarbonyl-alanyl-, 3′,5′-bis-O-N-α-benzyloxycarbonyl-valyl ,3′,5′-bis-O-N-α-benzyloxy-carbonyl-glycyl-glycyl-glycyl,3′,5′-bis-O-N-α-benzyloxycarbonyl-phenylalanyl,3′,5′-bis-O-N-α-benzyloxycarbonyl-glycyl) were calculated at the semiempirical (AM1) level. The choice of method is limited by the molecular size. In addition, the differences between the ground state energy of the compounds and that of the hydrolysis reaction intermediates were compared to the experimentally found stability towards hydrolysis.

This investigation was performed in search of new 2′-deoxynucleoside analogues modified at 3′- and 5′-positions with amino acids and possessing antiviral activity.

Substrate mimetics strategy: An efficient approach to protease-catalyzed peptide ligation

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Two main drawbacks seriously restrict the synthetic value of proteases as reagents in peptide fragment coupling: (1) native proteolytic activity and, thus, risk of undesired peptide cleavage; (ii) limited enzyme specificities restricting the amino acid residues between which a peptide bond can be formed. The latter can be overcome by the use of substrate mimetics. Contrary to common acyl donors, substrate mimetics bear a binding site specific ester leaving group instead of having a specific amino acid moiety at the C-terminus of the acyl residue. This replacement mediates the acylation of the protease by non-specific acyl residues. Deacylation of the artificial acyl enzyme intermediate by the amino component added results in peptide bond formation regardless of the primary specificity of proteases enabling nonspecific coded and noncoded amino acid derivatives and even non-amino acid-derived acyl moieties to be coupled. The successful application of these artificial substrates for model peptide ligations catalyzed by the Arg-specific trypsin, the Glu-specific *Staphylococcus aureus* strain V8 protease (V8 protease), and α-chymotrypsin, which is specific for aromatic amino acid moieties, will be demonstrated.

New development in the tritium labelling of peptides and proteins using solid state catalytic isotopic exchange with spillover-tritium

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[1] Institute of Molecular Genetics, Russian Academy of Sciences, and
[2] Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russia

The reaction of high temperature solid state catalytic isotope exchange (*HSCIE*) of hydrogen in peptides and proteins with spillover-tritium was studied. The reaction ability of amino fragments in *HSCIE* was shown to depend both of their structure and on the availability and the mobility of the polypeptide chain. [^{3}H] peptide analysis using ^{3}H NMR spectroscopy was carried out, and the modified fragment [^{3}H]ACTC$_{4–10}$ (Met-Glu-His-Phe-Gly-Pro), with molar activity of 80 Ci/mmol and [^{3}H] zervamicin IIB (Ac-Trp-Ile-Gln-Iva-Ile-Trh-Aib-Leu-Aib-Leu-Hyp-Gln-Aib-Hip-Aib-Pro-Phl, where Aib = 2-amino-isobutyric acid) with molar activity of 70 Ci/mmol was produced. The obtained preparations completely retained their biological activity.

With the β-galactosidase protein from *Termoanaerobacter ethanolicus* as example, the interrelation between the protein's tertiary structure and the isotopic label distribution incorporated due to the *HSCIE* reaction was used. The labeled protein with the molecular mass of 83 kDa was brought to fragmentation by Glu-proteinase. Peptide fragments were separated by HPLC and were identified by MALDI mass spectrometry. A correlation between the position of the amino acid fragment in the protein tertiary structure and its reaction ability in the *HSCIE* reaction was obtained. Data on the retention of the β-galactosidase enzymatic activity in condition of tritium label introduction are supplied.

Taurine

Taurine chloramine modulates cytokine production by peripheral blood mononuclear cells

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Objective. Proinflammatory cytokines are produced in a cascade fashion, where monocyte-derived TNFα and IL-1β trigger production of IL-6 and IL-8 also in the other cell types. We reported recently that taurine chloramine (Tau-Cl) inhibits production of the latter cytokines in fibroblast-like synoviocytes. In present study the effect of taurine (Tau) and Tau-Cl on TNFα, IL-1β and IL-6 production was examined.

Methods. Peripheral blood mononuclear cells from healthy volunteers were stimulated with LPS (24 h) in the presence of Tau or Tau-Cl (100–500 μM). Cytokine production was measured in culture supernatants (secreted) and cell lysates (intracellular) using ELISA.

Results. In LPS-stimulated cells both secreted and intracellular IL-1β and IL-6 were inhibited by Tau-Cl with $IC_{50} \approx 300\,\mu M$ and 425 μM, respectively. However, Tau-Cl exerted dual effect on TNFα production, raising it slightly (1.5 times) at low (100–200 μM) while reducing it ($IC_{50} \approx 450\,\mu M$) at higher concentration. Tau did not significantly affect cytokine production.

Conclusion. Tau-Cl modulates proinflammatory cytokine cascade and eventually might down-regulate it when present at high (>300 μM) concentration.

Thyrotropin- and swelling-activation of a taurine efflux pathway in rat thyrocytes is mediated by cyclic AMP

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Every living cell must deal with osmotic and hydrostatic pressure changes between its environment and its interior and counteract volume changes. Swelling activated channels is one group of effectors in the cell membrane that is important in preventing excessive volume increases by releasing inorganic ions and organic solutes that include taurine. Such channels are associated with several physiological processes, but little is known about their activation mechanisms.

We have used a rat thyroid cell line (FRTL-5) to investigate the activation of a swelling sensitive [3H]taurine efflux pathway. Hypo-osmolality and thyrotropin (TSH, 500 μM) increased transiently the rate coefficient for [3H]taurine efflux with a similar pattern of activation. The phosphodiesterase inhibitor 3-isobutyl-1-methyl xanthine (100 μM) increased the swelling activated efflux rate coefficient 6.4 times above the

control level and the cAMP analogue dibutyryl-cAMP (500 μM) activated the pathway. These results indicate that both swelling and TSH activation of the taurine efflux pathway are mediated by cAMP. Other aspects of the signal transduction pathway will be discussed.

N, N-Dichlorotaurine: chemical and bactericidal properties

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Based on the inclination of *N*-chloroamines to disproportionate, the endogenous bactericidal agent N-chlorotaurine (NCT), mainly at pH < 7, is accompanied by N, N-dichlorotaurine (NDCT). Since pure NDCT could be synthesized as crystalline sodium salt, a first evaluation of its chemical and bactericidal properties was possible.

NDCT-Na (melting point: 162–4°C, decomp.) is very well soluble in water and poorly in ethanol where it can be recrystallized from. On storage the initial pH 5 of the aqueous solution decreases which correlates with a decrease of oxidation capacity of 1.8% per day, probably originated by the elimination reaction $R—CH_2—NCl_2 \rightarrow R—CH=NCl + H^+ + Cl^-$ as a first step. Contrary to NCT-Na an immediate decomposition occurs when NDCT-Na comes into contact with undiluted DMSO. In aqueous solution, however, NDCT does not react with DMSO.

The bactericidal activity of 55 mM NDCT at pH 7 and 5 against the Gram-positive bacteria *S. epidermidiand two strains of S. aureus* was the same as with equimolar NCT though NDCT bears twice the oxidation capacity. Against the Gram-negative bacteria *E. coli*, *P. aeruginosa*, and *P. mirabilis*, however, a significantly higher activity of NDCT was observed at both pH.

The mechanism of taurine chloramine inhibition of fibroblast-like synoviocytes growth

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Objective. In rheumatoid arthritis (RA) enhanced proliferation of fibroblast-like synoviocytes (FLS) leads to hyperplasia of synovial membrane (SM). Therapeutic approaches to inhibit an excessive growth of these cells are not satisfactory. Thus, we investigated the effect of taurine (Tau) or taurine chloramine (Tau-Cl) on RA FLS growth.

Methods. FLS isolated from SM of RA patients were stimulated for 72 hours with rhPDGF or rhTNF-α. Tau or Tau-Cl were added at 100–250 μM concentrations. Cell proliferation was determined by incorporation of ^{3}H-thymidine into DNA. Expression of proteins regulating cell-cycle progression or apoptosis, was estimated by Western blotting.

Results. At 250 μM concentration Tau-Cl inhibited (by ≈ 70%) both PDGF- and TNF-α-triggered cell proliferation and similarly reduced expression of PCNA (a cofactor for DNA polimerase δ). However, Tau-Cl affected neither the expression of cell-cycle inhibitors (p21, p27) nor anti-apoptotic Bcl-2 protein. Tau has no effect on tested responses.

Conclusion. We report that Tau-Cl inhibits proliferation of RA FLS by affecting expression of PCNA, that is critical for cell cycle progression.

Taurine chloramine inhibits transcription of cytokine (IL-6, IL-8) genes via down-regulation of NFκB and AP-1 transcription factor activities

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Objective. Proinflammatory cytokines play critical role in the pathogenesis of rheumatoid arthritis (RA). We reported recently that taurine chloramine (Tau-Cl), but not taurine (Tau), inhibits production of IL-6 and IL-8 by fibroblast-like synoviocytes (FLS). In present study the mechanism of Tau-Cl inhibitory action was investigated.

Methods. FLS isolated from synovial membrane of RA patients were stimulated with rhIL-1β. Tau or Tau-Cl were added at 250–500 μM concentration. After 0.5–2 h or 4 h the DNA binding activity of NFκB and AP-1 (EMSA) and the expression of IL-6 and IL-8 mRNAs (RT-PCR) was examined, respectively.

Results. IL-1β raised NFκB and AP-1 activity, followed by the elevation of cytokine mRNAs expression. Tau-Cl, but not Tau, reduced both the expression of cytokine mRNAs (IL-6 > IL-8) and the activity of transcription factors (NFκB > AP-1).

Conclusion. Tau-Cl inhibits transcription of IL-6 and IL-8 genes due to its ability to diminish the activity of key transcriptional factors, that regulate these proinflammatory cytokine expression.

MRS studies on taurine in rat brain: Biosynthesis and *in-vivo* detection

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The synthesis of taurine and hypotaurine from cysteine can be followed up in astroglia-rich primary cultures obtained from brain of neonatal Wistar rats. Using ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy cell extracts of glia cells incubated with ^{13}C labelled cystein show the label subsequently in hypotaurine, taurine, lactate and gluthathione. Within 72 h, 35% of the total intracellular hypotaurine and 22.5% of taurine were newly synthesized from cysteine. Both metabolites were also released to the medium. Neurones are capable to take up both metabolites from glia media to recruit their organic osmolite. Part of newly synthesized glutathione and lactate are also exported to the medium. By this means lactate may serve as an energy substrate for neurons.

In-vivo MRS of lactate is obscured by line splitting and signal overlay. Using various two dimensional pulse sequences as spin preparation sequences prior to localized single voxel, in-vivo MRS or spectroscopic imaging sequences will provide homonuclear non-coupled resonance signal of taurine. These singlet signals are detectable and quantified. Diffusion weighted spectroscopy is used to characterize the mobility of taurine in living tissue.

Taurine depletion in the mammalian cell – physiologic ramifications

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Taurine depletion, whether by removing taurine from the diet or by using a taurine transport inhibitor, has demonstrated

various pathologies in various animal models including man. The first reported pathology associated with dietary taurine depletion was in the retina of the cat. In this animal model, taurine deficiency resulted in disorganization of the tapetum (the light reflecting membrane), disruption of the outer segments, photoreceptor dysfunction, and cell loss. When allowed to proceed for a number of months the result was blindness. Subsequent studies demonstrated that taurine deficiency also had a profound effect on cardiac physiology. Echocardiograms of the left ventricle of the cat heart depleted in taurine showed a dilated cardiomyopathy reflected in an extended end-diastolic diameter and an extended end-systolic diameter. Dietary taurine supplementation resulted in the above parameters returning to normal. The cat is a difficult animal model to use for a variety of reasons and thus the rat was chosen to further probe the consequences of taurine depletion. Unfortunately, the tissue taurine levels in the rat do not respond to dietary taurine depletion, and thus other experimental means had to be designed. Guanidinoethanesulfonic acid (GES), an analogue of taurine and a taurine transport inhibitor, has been utilized for the last 20 years to deplete rat tissues of their taurine content (J. E. Shaffer and J. J. Kocsis, Methods of reducing tissue taurine levels, and R. J. Huxtable, H. E. Laird, and S. Lippincott, Rapid depletion of tissue taurine content by guanidinoethyl sulfonate. In: The Effects of Taurine on Excitable Tissues, Spectrum Publications, New York, 1981). GES, when administered to rats in their diet in the drinking water as a 1–1.5% solution, usually produces a significant decrease in the taurine content of all tissues within one week of treatment. Within 3–5 weeks the levels of taurine reach their nadir (20–50% of control) and continued feeding of GES does not further reduce the levels of taurine. Unfortunately, GES replaces taurine and thus one must always consider the effects of GES on physiological events that occur within the tissues in question.

Again, as in the cat, taurine depletion manifested itself in retinal pathology: disruption of the photoreceptor structure, dissociation of the disc membranes, and abnormal electroretinograms (ERG). Other animals models such as the monkey have also demonstrated structural disorganization of the photoreceptors and abnormal ERGs. Finally, the ultimate test is whether taurine deficiency has an effect in man. In 1985, Koppel and associates (Geggel et al., N. Eng. J. Med. 312: 142–146, 1985) demonstrated that children on long term parenteral nutrition devoid of taurine had abnormal ERG. Supplementation of the parenteral nutrition with taurine restored the ERGs to normal in the majority of the children. Because of these definitive studies, all infant formulae in the United States, Europe and Japan now contain taurine.

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Effects of taurine depletion on protein phosphorylation in cardiac tissue

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It has been demonstrated previously in our laboratory that taurine inhibits the phosphorylation of an ~44 kdalton protein present in the mitochondrial fraction of the rat heart (J. Mol. Cell. Cardiol. 26: 1675–1689, 1994). Upon administering 1.5% guanidinoethanesulfonic acid (GES) in the drinking water of rats for 6 weeks, the taurine levels in cardiac tissue decline by 75%. However, the phosphorylation of a ~44 kdalton protein in the mitochondrial fraction of the heart tissue increased by 85% (J. Mol. Cell. Cardiol. 26: 1675–1689, 1994). Reversal of these effects could be accomplished by feeding the rat 1.5% taurine in the drinking water for 6 weeks. The ~44 kdalton protein was isolated by 1-dimensional polyacrylamide gel electrophoresis (PAGE) using traditional glycine buffers followed by re-electrophoresing the cut out portion of the gel, which corresponds to the ~44 kdalton protein, on a tricine-buffered gel resulting in sufficient pure protein for digestion and sequence analysis. It was determined that the ~44 kdalton was pyruvate dehydrogenase (Amino Acids 12: 139–144, 1997) which indicates a significant regulatory role for taurine in energy metabolism in cardiac tissue. These data are of significant interest in that taurine may be an additional effector of this enzyme or of the enzyme complex. Studies are in progress to determine if taurine has a direct effect on either the kinase (inhibition) or the phosphatase (stimulation) associated with the pyruvate dehydrogenase complex.

It has also been demonstrated and now reported that taurine depletion utilizing GES in vivo in rats affects the phosphorylation of myelin basic protein (MBP). In these experiments the animals were given GES (1%) for 6 weeks in their water and then killed; the hearts were removed and homogenized. The homogenate was then incubated with buffer containing MBP (50ì) and radioactive ATP for 6 minutes. Animals were also treated with taurine (1%) in their drinking water for 4–5 weeks or treated with taurine following the GES treatment. PAGE of the incubation mixture, autoradiography on the dried gel, and densitometry of the MBP band gave the following results:

Relative % activity ± SEM (normalized to mg protein)

Control 100 ± 20 (N = 6)
GES-treated 147 ± 28 (N = 6) $P < 0.05$ (paired 5-test)

Control 100 ± 16 (N = 5)
Taurine-treated 95 ± 17 (N = 5) $P > 0.05$

Control 100 ± 32 (N = 4)
GES followed by Taurine 95 ± 37 (N = 4) $P > 0.05$

These data confirm previous reports that it is easier to deplete animals of their cardiac taurine content than it is to raise the levels of taurine. These data on the effects of taurine depletion (increase in MAPkinase activity) and taurine supplementation (no change in MAPkinase activity) on MAPkinase activity reflect these past observations.

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Effects of propofol on neutrophil (PMN) free amino acid profiles and immune functions *in vitro*. Immunologic and metabolic consequences of taurine supplementation

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We have been examined the effects of propofol, taurine and propofol combined with taurine on free intracellular amino acid (AA) profiles, superoxide anion formation (O_2^-), hydrogen peroxide production (H_2O_2) and released myeloperoxidase activity (MPO) in polymorphonuclear leucocytes (PMN). Propofol led to significant changes in PMN free taurine, glutamine, glutamate, aspartate, methionine, basic, neutral (NAA) and branched chain amino acid concentrations. Exogenous taurine reduced PMN NAA while increasing intracellular taurine. Taurine supplemented to propofol significantly reversed the changes in taurine, NAA and alanine only. Regarding PMN immune functions propofol significantly decreased O_2^-, H_2O_2 formation and MPO. Taurine decreased O_2^- and H_2O_2 production, while increasing released MPO. When propofol and taurine were combined they appeared to

act additively, or in the case of MPO negated each other's effects. Regarding our results there is significance to pharmacological regimens which enhance the supply of propofol or taurine in whole blood. These regimens influence considerably PMN intracellular amino acid concentrations and it is this PMN "labile free amino acid pool" which may be one of the determinants in cell nutrition positively or adversely affecting PMN immune functions. Taurine supplementation to PMN seems to interfere independently from the effects of propofol on PMN free amino acids and on immune functions tested.

Interaction of N-chlorotaurine with amino acids and ammonium: enhancement of bactericidal activity and clinical consequences

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N-chlorotaurine (NCT) is a long-lived oxidant produced by activated human leukocytes during the oxidative burst. It has activity against a broad spectrum of pathogens including bacteria, fungi, viruses and helminths.

As a special feature, the killing of microbes by NCT can be increased significantly in the presence of ammonium and also of some amino acids (alanine, glycine). This is explained by transfer of the active chlorine ("transhalogenation") from NCT to ammonium and amino acids to form the corresponding, stronger microbicidal N-chloro derivatives monochloramine and N-chloro amino acids, respectively. Especially addition of ammonium to NCT provokes rapid inactivation of fungi and even mycobacteria.

Because of its good tolerability, NCT solution can be applied to human tissue to treat infections. In ammonium-containing body fluids like nasal mucus and urine, fungi and bacteria are killed within minutes. Therefore, amino compounds of human secretions can be transformed to the above quoted endogenous and highly microbicidal chloramines by NCT via transhalogenation – a unique property of an antimicrobial agent. Successful treatment in cases of urinary tract and otorhinolaryngological infections and conjunctivitis in phase IIa clinical trials provides strong support for this concept.

A role for taurine in ethanol sensitivity and ethanol-seeking behavior

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The endogenous sulfonated amino acid taurine has numerous functions in the central nervous system, including positive modulation of $GABA_A$ receptor function. Recently we found that mice lacking protein kinase C – epsilon (PKCε) are behaviorally and biochemically supersensitive to ethanol and other positive allosteric modulators of the $GABA_A$ receptor. In addition, these mice consume 50–75% less ethanol and wild-type controls in two separate self-administration paradigms. Microdialysis studies in PKCε-deficient mice revealed elevated extracellular levels of taurine, which may account for the supersensitivity of $GABA_A$ receptors in these mice and resulting decreases in ethanol intake. In light of the fact that the taurine derivative acamprosate (calcium acetylhornotaurinate) is moderately effective in reducing craving and relapse in detoxified alcoholics, we examined the effect of taurine-related compounds on acute ethanol consumption in a two-bottle choice paradigm in rats. Taurine (10–200 mg/kg IP) was only slightly effective in reducing ethanol intake but not preference, while the highest dose of taurine (200 mg/kg) also suppressed water intake. The taurine precursor hypotaurine (10–100 mg/kg IP) was also weakly effective in reducing ethanol intake but not preference or water intake. The most effective compound tested was homotaurine (10–100 mg/kg IP), which suppressed ethanol intake and preference by approximately 50% without altering water intake. These data indicate that endogenous taurine may regulate sensitivity to ethanol and subsequent ethanol self-administration, and that taurine-related compounds may be effective in reducing alcohol intake in humans. We are currently exploring whether taurine and related compounds are able to suppress ethanol-stimulated mesolimbic dopamine release, a primary neural substrate of ethanol reinforcement.

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Osmotic preconditioning through taurine depletion and treatment

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Organic osmolytes, such as taurine, regulate a cell's osmotic balance without directly altering either the cell's ionic composition or the membrane potential. This property of the organic osmolyte often renders the cell resistant to damage during a pathological insult. Indeed, ischemia is associated with a massive efflux of taurine from the cell, an event that minimizes the severity of the osmotic imbalance that develops from the accumulation of lactate, inorganic phosphate and sodium. However, taurine depletion also activates specific signaling pathways that provide further protection to the cell. Among the signaling pathways activated by taurine depletion is a PI 3-kinase (phosphatidylinositol 3-kinase) linked pathway that catalyzes the phosphorylation and inactivation of the pro-apoptotic factor, Bad. Taurine depletion also activates protein kinase C, which in turn elevates the intracellular content of the anti-apoptotic factor, Bcl-2. Increases in the extracellular osmolality by either addition of 20 mM taurine or 25 mM mannitol to the incubation medium activates similar pathways. However, PI 3-kinase assumes a more important role in the mannitol treated cell than the taurine depleted cell. Moreover, p38 MAP kinase is activated by mannitol treatment but not by taurine depletion. Despite these differences, both taurine depletion and mannitol treatment protect the cell against hypoxia-induced apoptosis. The data suggest that osmotic stress protects the cell against apoptosis by increasing cellular levels of Bcl-2 and promoting the inactivation of Bad. This work was supported by a grant from the American Heart Association.

The regulation of sulphurated amino acids connection. A dummy or a protagonist on the stage of inflammation?

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Amino Acids are usually present in large excess in healthy and the excess is used as source of calories. However, metabolic alterations are observed in ill patients and preferential retention of Sulphur Amino Acids (SAA) occurs during the inflammatory response. The metabolism of Cysteine is modified during the acute phase of sepsis in rats. Sulphate production is lower, whereas the higher liver production of Taurine seems to play a protective role; Glutathione concentration is greater in liver, kidney and other organs and Cysteine incorporation into proteins was higher in spleen, lung and plasma (Acute Phase Proteins) while Albumin level decreases.

Another important phenomenon is the impairment of Methionine conversion to Cysteine during stressed condition. Premature infants or HIV patients synthesise Cysteine from Methionine at a much lower rate. Thus, the metabolic flow through the trans-sulphuration pathway may be insufficient to meet the Glutathione and Cysteine requirement in critical conditions. The pro-inflammatory cytokines, Interleukin-1, Interleukin-6 and TNF-α are the main initiates that alter protein and amino acid metabolism. In this complex picture, SAA supply may contribute to the immune system regulation.

Regulation of the taurine transporter and the taurine biosynthetic enzymes in liver cells

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The intracellular level of taurine is maintained not only by the taurine transporter that transports extracellular taurine inside cells but also by endogenous synthesis from methionine and cysteine. We therefore investigated the regulation of both the taurine transporter and the cysteine dioxygenase, one of the main taurine biosynthetic enzymes, in HepG2 human liver cells. The intracellular taurine content of HepG2 cells was extremely increased by culturing in a hypertonic medium. The activity of taurine transport was increased by hypertonic conditions, which was due to the increased expression of the taurine transporter gene. The expression level of the cysteine dioxygenase gene was also increased, suggesting that the expression levels of both the taurine transporter gene and the cysteine dioxygenase gene were regulated in harmony by hypertonic conditions to accumulate taurine inside cells. On the other hand, the activity of taurine transport in HepG2 cells was down-regulated on culturing the cells in taurine-rich medium, the expression level of the taurine transporter gene being also markedly decreased. However, the expression level of the cysteine dioxygenase gene was not significantly altered under taurine-rich conditions, indicating that the gene expression of the taurine transporter and that of the cysteine dioxygenase was independently regulated by extracellular concentration of taurine.

Protein kinase C distribution: Mechanism underlying the interaction between taurine and angiotensin II

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The amino acid, taurine, is found in very high concentration in the heart. Although its most important putative function is osmoregulation, it also serves as a regulator of cell growth. Isolated cardiomyocytes exposed to medium containing 1 nM angiotensin II undergo hypertrophy, a process blocked by 20 mM taurine. The amino acid also inhibits angiotensin II-induced activation of c-fos, upregulation of atrial natriuretic factor and induction of TGF-betal. Central to virtually all of these actions of angiotensin II is the translocation and activation of key protein kinase C (PKC) isoforms. Therefore, we proposed that taurine inhibited the hypertrophic actions of angiotensin II by interfering with the translocation of one or more of the PKC isoforms. Indeed, taurine and angiotensin II exhibited different effects on the translocation of several PKC isoforms. While taurine promoted the translocation of PKCalpha, PKCdelta and PKCepsilon from the particulate fraction to the cytosol, the levels of the three isoforms in the particulate fraction were elevated following treatment with angiotensin II. By contrast, both taurine and angiotensin II increased the PKCzeta content of the particulate fraction and the PKCbeta2 content of the cytosol. When the isolated cardiomyocytes were incubated with medium containing both angiotensin II and taurine, the effects on PKC distribution were largely additive. These data support the notion that taurine prevents the hypertrophic effects of angiotensin II by interfering with the translocation of either PKCalpha, PKCdelta, PKCepsilon or a combination of more than one of the isoforms.

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Excretion of sulfate and taurine in human urine

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Main final metabolites of L-cysteine in mammals are sulfate and taurine, and they are excreted in the urine. Our previous studies in rats have shown that the ratio of urinary sulfate and taurine in rats fed diet containing sufficient methionine and cysteine is 10: 2–3.

In the present study, we determined urinary sulfate and taurine in urine samples of 58 healthy Japanese women after 12-h starvation following usual meal. Free (inorganic) and total (free + ester) sulfate were determined with ion chromatography, and taurine by reversed-phase HPLC after dabsylation. Average excretions (micromols per mg of creatinine) were: total sulfate, 12.53 ± 3.85; free sulfate, 11.57 ± 3.69; ester sulfate, 0.96 ± 0.94; taurine, 0.78 ± 0.53; urea, 187.71 ± 66.13. The ratio of total sulfate and taurine was 10 : 0.62. This suggests that sulfate formation in humans is more dominant than taurine formation as in rats and this tendency is more evident in humans than in rats, which is in accordance with low cysteinesulfinate decarboxylase activity in humans. Sum of sulfate and taurine excretions was significantly correlated with that of urea: correlation coefficient, 0.675. This indicates that sulfur metabolism in humans is in the state of sulfur equilibrium similar to that of nitrogen and reflects protein metabolism.

Dietary taurine enhances cholesterol degradation and reduces serum and liver cholesterol concentrations in rats fed a high-cholesterol diet

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The effect of taurine on hypercholesterolemia induced by feeding a high-cholesterol (HC) diet to rats was examined. When various amounts of taurine (0.25–50 g/kg) were supplemented to HC for 2 wk, serum total cholesterol gradually and significantly decreased in a dose-dependent manner, compared with the control (cholesterol free) diet group. By contrast, serum HDL-cholesterol was elevated by taurine supplementation. In the hypercholesterolemic rats fed the HC diet, the excretion of fecal bile acids and hepatic cholesterol 7α-hydroxylase (CYP7A1) activity and its mRNA level increased significantly, and the supplementation of taurine further enhances

these indexes, indicating an increase in cholesterol degradation. Agarose gel electrophoresis revealed that, in hypercholesterolemic rats fed the HC diet, the serum level of the heavier VLDL increased significantly, but taurine repressed this increase and normalized this pattern. Significant correlations were observed between the time- and dose-dependent increases of CYP7A1 gene expression and the decrease of blood cholesterol concentration in rats fed the HC diet supplemented with taurine. These results suggest that the hypocholesterolemic effects of taurine observed in the hypercholesterolemic rats fed the HC diet were mainly due to the enhancement of cholesterol degradation and the excretion of bile acid.

Mechanism of ammonia-induced taurine accumulation in the rat striatum *in vivo*

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In vitro studies have shown that ammonia, which is responsible for neurological symptoms associated with hyperamonemia, causes a massive release of taurine from cultured CNS cells and brain slices. In this study, taurine (Tau) release was measured in vivo in rat striatum following direct application to the microdialysis tube of 60 mM ammonium chloride which renders the final ammonia concentration in the extracellular space of ~5 mM. Various *in vivo* stimuli evoke taurine efflux either by opening osmosensitive anion channels and/or by a mechanism secondary to Glu accumulation and its interaction with NMDA or AMPA receptors. The following compounds were coadministered with ammonia to distinguish between these mechanisms: anion/cation transport inhibitors – DIDS and furosemide, a Glu transport inhibitor-PDC, and NMDA and AMPA/KA receptor antagonists dizocilpine and DNQX. Ammonia stimulated Tau accumulation in the microdialysates to ~250% of basal value. DIDS and furosemide moderately inhibited the effect of ammonia (furosemide by ~30%), albeit DIDS added alone induced massive accumulation of Tau with a delayed onset as compared to ammonia. Ammonia-dependent Tau accumulation was increased by ~50% in the presence of PDC and reduced in an equal degree (~35%) by dizocilpine and DNQX. None of the agents affected Tau accumulation in the absence of ammonia. The results show that ammonia in vivo evokes Tau accumulation both via anion channels, possibly secondary to cell volume changes, and in consequence of stimulation of both NMDA and AMPA/KA receptors.

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Amino Acids Transport

Transductional regulation of expression and function of cationic amino acid transporters

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The discovery in 1987 that endothelium-derived relaxing factor is nitric oxide (NO) was followed a year later with reports that the cationic amino acid L-arginine is the physiological precursor for nitric oxide. It has since been established that the terminal guanidinium nitrogen of L-arginine is metabolised via a series of oxidation reactions resulting in NO production, with citrulline being formed as a co-product. Of interest was the parallel observation that uptake of L-arginine was enhanced in iNOS expressing cells and that this was due to *de novo* synthesis of carrier proteins. The precise signaling pathway that regulates the enhanced expression of these carriers has been the subject of intense studies in recent years. Current literature suggests that activation of upstream signaling molecules such as protein kinase C may be critical. In addition, downstream kinases thought to be points of convergence for various signals originating from cell surface receptors have also been implicated. Two of these downstream targets include the 42 and 44 kDa forms of mitogen-activated protein kinase (MAPK) and the stress-activated 38 kDa MAPK. It is worth noting however that the involvement of these different transduction pathways in the regulation of the induction of L-arginine transporters is not universal, and likely to be different from system to system. As a result there has been conflicting data on the relevance of these signaling proteins in inducing L-arginine transport in different cell. These issues will be discussed and the individual signaling pathways assessed on a cell type and species basis. Moreover, the role of downstream signaling molecules will be examined in more detail, looking in particular at the critical dependency on the p38 MAPK. This kinase currently exists in four different isoforms which are p38α, β, γ and δ. The involvement of individual isoforms of p38 in enhancing the expression of carrier proteins for L-arginine will be discussed.

Transport mechanisms for GW274150, a potent and selective inhibitor of inducible nitric oxide synthase

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GW274150 is an acetamidine derivative of hetero-substituted lysine which has been shown to have a marked selectivity for the human inducible nitric oxide synthase isoform (Young et al. 2000. Bioorg. Med. Chem. Lett., 10: 6, 597–600). The systems associated with transport of this compound have been investigated using the macrophage cell line J774. Prior to each study, J774 cells were seeded in 96-well culture plates and allowed to adhere for 24 h in Dulbecco's modified Eagle's medium (DMEM). Transport studies were carried out using Hepes buffered Krebs solution (50 µl; 37°C) containing L-[^{14}C]GW274150 (1 µCiml^{-1}) in the presence of either 0.1 mM or 0.025–1 mM unlabelled substrate. In parallel studies transport (1 µCiml^{-1}, 0.1 mM) was monitored in the presence of 1 mM excess of various other amino acids known to be substrates for distinct transport systems.

Time course experiments revealed that transport of 0.1 mM of L-[^{14}C]GW274150 occurred in a time-dependent manner and was linear for up to 5 min. In addition, uptake was only marginally dependent on extracellular Na^+. Kinetic studies revealed that transport was saturable, and Michaelis-Menten analysis revealed single affinity entry with an apparent K_t of 0.31 mM and V_{max} of 5.15 pmol·µg protein^{-1} min^{-1}.

At 1 mM, 2-methylaminoisobutyric acid (MeAIB), L-alanine, L-valine and β-2-amino-bicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) caused little or no inhibition of L-[^{14}C]GW474150 (0.1 mM) uptake. In contrast, transport of L-[^{14}C]GW274150 was inhibited markedly by L-arginine, L-lysine, L-leucine, L-methionine, 6-diazo-5-oxo-L-norleucine (DON) and L-glutamine. With the exception of L-arginine and L-lysine, the inhibition caused by the other substrates was critically dependent on extracellular Na^+ and was completely reversed when extracellular Na^+ was replaced with choline. In parallel kinetic inhibition experiments, transport of 0.1 mM L-[^{14}C]GW274150 was inhibited in a concentration dependent manner by L-arginine (Ki = 0.04 mM), L-leucine (Ki = 0.06), DON (Ki = 0.18 mM) and L-glutamine (Ki = 0.13 mM).

Taken together, these data suggest that GW274150 may be transported, at least in part, by system y^+. However, the marked inhibition caused by L-leucine, L-glutamine and L-methionine, substrates for the relatively high affinity cationic amino acid transporter system y^+L, would suggest that this system may also contribute to the uptake of GW274150; if so, the monophasic substrate kinetics imply that the two systems handle GW274150 with similar affinity. Other systems such as $B^{0,+}$ could be ruled out on the grounds that this transporter is critically Na^+-dependent while uptake of GW274150 is largely (~80%) Na^+-independent. Similarly, $b^{0,+}$, another broad-spectrum aminio acid transporter that may be capable of transporting GW274150 does not interact with L-glutamine and thus unlikely to be involved in transport of GW274150, at least in J774 cells.

Identification and characterization of the first mammalian electrogenic proton dependent amino acid transporter

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Although a large number of different amino acid transporters have been identified on a molecular basis, some of them – functionally described in mammalian cells – are still missing. In search of mammalian EST sequences, which contain the signature of the AAAP (amino acid/auxin permease) family, we identified a murine full length cDNA, which encodes a membrane protein with 10–11 putative transmembrane domains. The transporter mRNA is expressed in various murine tissues, including lung, heart and kidney. For functional characterization we used the *Xenopus laevis* oocyte expression system and employed flux studies and electrophysiological analysis. Oocytes injected with the cRNA showed an increased uptake of ^{3}H-L-alanine and ^{3}H-L-proline. Detailed electrophysiological analysis revealed an electrogenic transport mode, independent of sodium and chloride ions. Lowering the extracellular pH increased significantly substrate induced currents in cRNA injected oocytes. Out of the 20 proteinogenic amino acids the transporter recognizes only small amino acids, such as Gly, Ala, Pro and Ser. Distinct structural analogues of these amino acids also interact with the transporters substrate binding site. In conclusion, we describe the molecular and functional characteristics of the first electrogenic proton driven amino acid transporter of mammals.

Neuroactive components of medicinal herbs and neuronal amino acid transport: A neglected possibility?

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It is now well established that transport of amino acid neurotransmitters (like glutamate, aspartate, GABA and glycine etc.) from and to the neurones is essential for their proper functioning. Like in the case of other neurotransmitters, specific pre- and post-synaptic as well as vesicular transporters are involved in such processes. Extensive efforts to clarify the mechanisms and processes involved in the control and/or proper functioning of the amino acid transporters are now, therefore, being made in numerous laboratories. Such efforts have not only led to the identification of a few specific ligands and/or modulators of neuronal amino acid transporters, but also have started unravelling the complex and diverse processes regulating their functions.

Aim of this communication is to point out potential usefulness of some neuroactive constituents isolated from therapeutically used medicinal herbs for clarifying the mechanisms involved in neuronal amino acid transport. Our interest in such studies was initially triggered by the observations made with hyperforin, i.e. quantitatively the major neuroactive component of *Hypericum perforatum* extracts widely used for the treatment of mild to moderate depressive disorders. This acyl phloglucinol derivative not only modulate synaptic transports of biogenic amines but also of glutamate, aspartate and GABA. Since it does not interact with any of the till now described transporters for these neurotransmitters, efforts were made to clarify the mechanisms involved in their observed effects (both in vitro and as well as in vivo). The results of the in vitro studies available to date strongly suggest that its effects on neuronal amino acid transport processes is mediated via some novel extracellular mechanism controlling the H^+ (and/or other ionic) concentrations of neurones. These observations not only demonstrate that hyperforin represent a structurally and mechanistically novel class of therapeutically useful agent but also suggest that it could be useful tool for clarifying the complex mechanisms involved in the control of neuronal amino acid transport.

These observations stimulated us to screen other putative psychoactive herbal extracts and their active constituents on neuronal amino acid transport and on the consequences of disturbances caused by malfunction of specific transporters. Observations made with several such agents indicate that either modulation of mechanisms and/or processes involved in neuronal amino acid transport or reversal of pathologies caused by anomaly of transporter functions could be involved in their modes of actions. These observations reinforce our conviction that studies directed towards clarifying the effects of herbal constituents on neuronal amino acid transport might not only be a feasible way for identifying novel types of therapeutically interesting molecules but also could expedite our knowledge on these complex processes.

Glutamate-regulated sodium dynamics in cortical astrocytes: Implications for cellular bioenergetics

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The mode of Na^+ entry and the dynamics of intracellular Na^+ concentration (Na^+_i) changes consecutive to the application of the neurotransmitter glutamate were investigated in mouse cortical astrocytes in primary culture by video fluorescence microscopy. An elevation of Na^+_i was evoked

by glutamate, whose amplitude and initial rate were concentration-dependent. The glutamate-evoked Na^+ increase was primarily due to Na^+-glutamate cotransport. The rate of Na^+ influx decreased during glutamate application, with kinetics that correlate well with the increase in Na^+_i and which depend on the extracellular concentration of glutamate. A tight coupling between Na^+ entry and Na^+/K^+ ATPase activity was revealed by the massive Na^+_i increase evoked by glutamate when pump activity was inhibited by ouabain. During prolonged glutamate application, Na^+_i remains elevated at a new steady-state where Na^+ influx through the transporter matches Na^+ extrusion through the Na^+/K^+ ATPase. A mathematical model of the dynamics of Na^+_i homeostasis will be presented which precisely defines the critical role of Na^+ influx kinetics on the establishment of the elevated steady-state and its consequences on the cellular bioenergetics. Indeed, extracellular glutamate concentrations as low as 10 μM approximately doubled the energetic demands of the astrocytes.

Heteromeric amino acid transporters: From disease to structure

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In the last 5 years a new family of amino acid transporters composed by two different subunits has been defined. Two heavy subunits (rBAT and 4F2hc) and seven light subunits are known. rBAT and the light subunits b0,+ AT and y + LAT1 are responsible for the inherited aminoaciduria type I cystinuria, non-type I cystinuria and lysinuric protein intolerance, respectively. The heavy subunits are highly glycosylated type II proteins, while light subunits are very hydrophobic unglycosylated membrane proteins, displaying a polytopic (generally 12 transmembrane domains) predicted structure. The specificity of the amino acid transport activity depends on the light chain expressed. This, together with its topology, indicates that the transport function mainly relies on the light subunits.

I will summarize some of our current studies directed to the understanding of structure-function relationships of these heteromeric carriers, specially concerning their oligomeric structure and initial attempts to reconstitute them. Ongoing work on the isolation of new rBAT-associated light subunits and new b0,+ AT-associated heavy subunits, which could also play a role in cystinuria, will also be discussed.

Lessons from old and new members of the family of human cationic amino acid transporters (HCAT)

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Mammalian cationic amino acid transporters (CATs) catalyze the transport of basic amino acids through the plasma membrane. The CAT family comprises at least five related carrier proteins (CAT-1, -2A, -2B, -3 and -4) with CAT-2A and -2B being splice variants. In humans, only the "old" members of the family have been characterized (hCAT-1, -2A and -2B). hCAT-1 and -2B exhibit high affinity for cationic amino acids and are sensitive to *trans*-stimulation, consistent with the classical system y^+. In contrast, hCAT-2A is a low affinity carrier relatively insensitive to *trans*-stimulation. Interestingly, hCAT-2A and hCAT-2B differ only in a region of 42 amino acids. CAT-3, so far only identified in rat and mouse, exhibits also system y^+ activity. However, the substrate recognition and maximal transport activity seems to differ from other y^+ transporters. CAT-3 expression has been reported to be restricted to the brain in adult animals. A cDNA encoding for human hCAT-4 has recently been isolated, however, the transport activity of hCAT-4 has not been characterized. When optimally aligned, the amino acid sequence of hCAT-4 shows only about 40% identity with the other hCAT isoforms. In contrast, the amino acid sequences of hCAT-1, -2(A or B) and -3 are about 60% identical.

To elucidate which amino acids are responsible for the difference in the transport properties of the hCAT proteins, we constructed chimeric proteins between hCAT-1 and hCAT-2A and performed site directed mutagenesis. Using this approach, we identified two amino acid residues that are responsible for the different transport properties of hCAT-2A compared to the high affinity CAT-isoforms. To characterize the human CAT-3, we cloned a cDNA encoding hCAT-3. When expressed in *Xenopus laevis* oocytes, hCAT-3 had a similar transport activity and affinity for L-arginine as hCAT-1 or -2B. hCAT-3-mediated L-arginine transport was trans-stimulated and independent of extracellular Na^+ ions. Expression studies demonstrated that hCAT-3 is not only expressed in different regions of the human brain, but also in peripheral tissues. To investigate if hCAT-4 also functions as an amino acid transporter, we measured the transport of cationic, neutral and acidic amino acids in *Xenopus laevis* oocytes expressing hCAT-4, but could not detect an transport activity for any substrate tested. A bright fluorescence could be detected in the plasma membrane of oocytes expressing hCAT-4 with the green fluorescent protein attached to the C-terminus. Therefore, hCAT-4 might either need a complementary protein to function as an amino acid transporter or serve as a transporter for a yet unidentified substrate.

Renal amino acid reabsorption in immature and adult rats as a sensitive marker of heavy metal-induced nephrotoxicity (PT, CR, TL)

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The effects of cis-platinum (CP; 0.6 mg/100 g b. wt. i. p.), sodium dichromate (Cr; 1 mg/100 g b. wt. s. c.) and Tl_2SO_4 (Tl, 2 mg/100 g b. wt. i. p.) on renal amino acid excretion and plasma amino acid composition were investigated in 10- (both sexes) and 55-day-old (female) anaesthetised Wistar rats (Han:Wist). On the basis of diuresis experiments on conscious rats (determination of urinary volume and protein excretion) the mentioned doses and times (1st day after Cr in both age groups and in 10-day-old rats after CP and 3rd day after CP in adult rats; 2nd [55-day-old rats] and 5th–6th day [10-day-old rats] after Tl) were found out to be optimal for the characterisation of amino acid transport after heavy metal poisoning. Interestingly, in conscious 10-day-old rats Cr nephrotoxicity is not detectable after 1 mg/100 g b. wt. whereas all of the other experimental groups showed nephrotoxic effects of Cr, Tl and CP in conscious rats. Urine volumes were lower, but not significantly, in anaesthetised immature rats, independently of the administered nephrotoxin. Glomerular filtration rate (GFR) is significantly lower in 10-day-old rats compared to adults. After CP, Cr and Tl GFR is significantly reduced only in adult rats and age differences disappeared nearly completely.

In principle the renal fractional excretion (FE_{AA}) of amino acids was distinctly higher in immature rats as a sign of lower amino acid reabsorption capacity. Nevertheless, the amino acid plasma concentrations were relatively high in immature control rats. However, both Cr and CP did not distinctly influence

amino acid plasma concentrations. But in both age groups the administration of Cr and CP significantly decreased amino acid reabsorption capacity (increase in FE_{AA}) as a sign of nephrotoxicity, most pronounced in adult rats after CP. On the other hand, after Tl, the FE of amino acids was distinctly higher only in adult rats as a sign of lower amino acid reabsorption capacity and, thus, as a sign of higher nephrotoxicity. In immature animals FE_{AA} was increased only for few amino acids. However, in both age groups Tl administration significantly decreased plasma amino acid concentrations, more pronounced in immature rats.

The investigation of renal amino acid handling confirmed:

(1) Cr, CP and Tl were more nephrotoxic in 55-day-old animals compared to immature rats as could be demonstrated previously using other parameters for nephrotoxicity testing.
(2) The extent of toxic effects of heavy metals on the kidney is related to the maturity of renal functions involved in the enrichment of the respective metal in renal tissue and in its toxicity mechanism.
(3) Changes in the fractional excretion of amino acids (reduction in renal amino acid reabsorption capacity, e.g. increase in FE_{AA}) and in amino acid plasma concentrations (especially decreases as a consequence of enhanced renal loss of amino acids) are early indicators of nephrotoxicity.
(4) Therefore, the determination of renal amino acid handling is a highly sensitive marker for nephrotoxicity testing, both in immature and in adult rats.

Synthesis and characterization of high-affinity inhibitors of the H^+/peptide transporter PEPT2

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The mammalian H^+/peptide cotransporter PEPT2 was initially identified in the brush border membrane of renal proximal tubular cells as a high affinity type PTR2-family member. Here we describe the synthesis and functional analysis of novel high affinity inhibitors for PEPT2 that will be useful in further studies on structure and functions. Starting from Lys[$Z(NO_2)$]-Pro a series of different lysine-containing dipeptide derivates were synthesized and studied for interaction with PEPT2 based on transport competition assays in *Pichia pastoris* yeast cells and in epithelial SKPT cells, both expressing PEPT2. The two-electrode-voltage-clamp technique in *X. laevis* oocytes expressing PEPT2 was used to determine whether the compounds are transported electrogenically or block the uptake of dipeptides. Synthesis and functional analysis of Lys-Lys derivates containing $Z(NO_2)$ side chain protections provided a set of inhibitors that reversibly inhibited the uptake of dipeptides by PEPT2 with K_i values as low as 10 nM. This is the highest affinity of a ligand of PEPT2 ever reported. Moreover, based on the structure-function relationship we can conclude that the spatial location of the ε-amino protecting group in a Lys containing dipeptide and its intramolecular distance from the alpha C-atom are key factors for the transformation of a substrate into an inhibitor of PEPT2.

Molecular cloning and functional characterization of ATA3, a novel subtype of the amino acid transport system A

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Recent molecular cloning studies have revealed that the amino acid transport system A consists of more than one subtype. Two different system A subtypes, called ATA1 and ATA2, have been cloned and functionally characterized. ATA1 is expressed primarily in the brain and placenta whereas ATA2 is expressed ubiquitously. Heterologous expression studies have shown that these two subtypes cannot be distinguished functionally based on substrate affinity nor substrate specificity. We have now cloned a third subtype of system A, designated ATA3. It is expressed primarily in the liver. Apart from the liver, detectable level of expression is noted only in the skeletal muscle. Interestingly, ATA3 can be easily differentiated from the other two subtypes of system A based on functional characteristics. We first isolated rat ATA3 cDNA from a skeletal muscle cDNA library using rat ATA2 cDNA as the probe. Rat ATA3 consists of 547 amino acids and exhibits a high degree of homology in amino acid sequence to rat ATA1 (47% identity) and rat ATA2 (57% identity). Interestingly, this new transporter also has a comparable degree of homology to SN1 and SN2, the two known subtypes of the amino acid transport system N. However, when expressed heterologously in *Xenopus laevis* oocytes, rat ATA3 transports α-(methylamino)isobutyric acid (MeAIB), a specific model substrate for system A, confirming that this transporter is definitely a subtype of system A. System N does not transport this system A model substrate. With two-microelectrode voltage-clamp technique, we have shown that exposure of rat ATA3-expressing oocytes to neutral, short-chain aliphatic amino acids induces inward currents. The amino acid-induced current is Na^+-dependent and pH-dependent. Analysis of the currents with alanine as the substrate has shown that $K_{0.5}$ for alanine (i.e., concentration of the amino acid yielding half-maximal current) is 4.2 ± 0.1 mM and that the Na^+: alanine stoichiometry is 1:1. Subsequently, we have cloned the human homolog of rat ATA3 from a liver cell line (HepG2) cDNA library. Human ATA3 also contains 547 amino acids and shows 88% identity in amino acid sequence with rat ATA3. The sequence identity of human ATA3 with human ATA1 and human ATA2 is 47% and 57%, respectively. The homology of human ATA3 with human SN1 and SN2 is also similar (56% and 51% identity, respectively). The gene coding for human ATA3 contains 16 exons and is located on chromosome 12p13. In the human, ATA3 is expressed almost exclusively in the liver. When expressed in mammalian cells heterologously, human ATA3 mediates the transport of neutral amino acids, including MeAIB, in a Na^+-dependent manner. Interestingly, while characterizing the function of this clone, we have uncovered a unique feature of this system A subtype. Human ATA3 is capable of mediating the transport of cationic amino acids. In fact, the affinity of human ATA3 for cationic amino acids is higher than for neutral amino acids. However, the human ATA3-mediated cationic amino acid transport is Na^+-independent. In this respect, ATA3 is similar to transport system y^+L that also transports neutral amino acids in a Na^+-coupled manner and cationic amino acids in a Na^+-independent manner. In contrast, ATA1 and ATA2 have not been shown to interact with cationic amino acids. In addition to this difference in substrate specificity, ATA3 also differs from ATA1 and ATA2 in substrate affinity. ATA1 and ATA2 interact with MeAIB with a K_t of ~0.3 mM whereas the affinity of ATA3 for this model substrate is comparatively at least 20-fold lower (K_t, ~8 mM). But, ATA3 interacts with arginine with a K_t value of 0.3 mM. Since liver does not express any of the previously known high affinity cationic amino acid transporters,

ATA3 is likely to provide the major route for the uptake of arginine in this tissue.

Transporters of L-DOPA in two clonal OK cell lines function as exchangers with neutral and basic amino acids

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The present study examined the nature and regulation of the L-DOPA transporter in two functionally different clonal subpopulations of opossum kidney (OK_{LC} and OK_{HC}) cells. The inward transfer of L-DOPA was largely promoted through an energy-dependent and sodium-insensitive transporter, though a minor component (~15%) was found to require extraceilular sodium. L-DOPA uptake was insensitive to MeAIB, but competitively inhibited by BHC (OK_{LC}, IC_{50} = 336 μM; OK_{HC}, IC_{50} = 439 μM). L- and D-neutral amino acids and basic amino acids markedly inhibited L-DOPA accumulation. L-DOPA, L-leucine, L-arginine, BHC or L-arginine plus BHC stimulated [^{14}C]-L-DOPA efflux. The accumulation of L-DOPA was significantly higher at an acidic pH, and incubation of cells with L-DOPA (100 μM) resulted in marked intracellular acidification. Modulators of PKA, PKG, PKC and PTK failed to affect the accumulation of L-DOPA. Only the Ca^{2+}/calmodulin inhibitors inhibited L-DOPA uptake. It is likely that system $B^{0,+}$ might be responsible for the sodium-dependent uptake of L-DOPA in OK cells, whereas sodium-independent uptake of L-DOPA may include systems $b^{0,+}$ and LAT2, the activation of which results in *trans*-stimulation of L-DOPA outward transfer. The *trans*-stimulation of L-DOPA inward transfer by an imposed H^+ gradient suggest that OK cells are provided with an L-DOPA-H^+ cotransport system.

Translational regulation of the mammalian arginine transporter cat1 by nutrients: A novel cell-defence mechanism of translational regulation via an ires element

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Amino acids are essential nutrients for cell growth and maintenance. The essential amino acids arginine and lysine, are mainly transported via the cationic amino acid transporter 1 protein (cat1). The regulation of translation of the cat1 mRNA during amino acid starvation was studied. An adaptive response to amino acid starvation and stress is a global decrease of protein synthesis, by phosphorylation of the translation initiation factor eIF2a. Translation of the transporter mRNA increases when eIF2a is phosphorylated, allowing synthesis of the essential for survival arginine/lysine transporter protein. The mechanism of increased translation of this mRNA involves the induction of activity of a uORF-containing internal ribosomal entry sequence (IRES). Translation of the uORF and phosphorylation of eIF2a are required for increased activity. We propose that eIF2a phosphorylation triggers translational attenuation within the uORF, converting a relatively inactive, to a high activity IRES. This study demonstrates that like yeast, mammalian cells have developed a sophisticated response to stress conditions: when expression of most genes decreases, synthesis of stress response proteins increases to support cell survival.

Amino acid transport, cell volume and the regulation of cell death

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Cell volume regulatory mechanisms participate in a wide variety of cellular functions including regulation of epithelial transport, excitability, hormone and transmitter release, metabolism, migration, cell proliferation and apoptotic cell death. Besides ion transport, polyols, betaine and glycerophosphorylcholine, cells utilize amino acids including taurine to balance extracellular osmolarity and regulate their volume. Cells counteract shrinkage by uptake and swelling by release of amino acids including taurine. Moreover, cell swelling stimulates synthesis and cell shrinkage favours breakdown of proteins which are osmotically less active than the sum of the amino acids thus generated.

Conversely, amino acid transport does influence cell volume. Concentrative uptake of amino acids leads to cell swelling, amino acid release to cell shrinkage. Through alterations of cell volume the amino acids participate in the regulation of protein metabolism. Thus, concentrative amino acid transport inhibits and release of amino acids favours proteolysis.

These mechanisms participate in the regulation of cell death. CD95 induced apoptotic death of Jurkat T lymphocytes is paralleled by the release of taurine. The taurine release occurs with a delay of some 60 min following CD95 receptor triggering but immediately preceedes apoptic cell shrinkage and DNA fragmentation. The signaling leading to taurine release is in large part elusive but requires at some stage activation of caspases. Moreover, taurine release and apoptotic DNA fragmentation are strongly inhibited by lowering of temperature. Preloading of the cells with taurine retards CD95 induced DNA fragmentation pointing to an active role of taurine in the regulation of apoptosis.

The knock-out of the peptide transporter gene PEP-2 results in delayed development and extended life-span in *Caenorhabditis elegans*

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Peptide transporters of the PTR-family are integral plasma membrane proteins, that mediate the electrogenic proton-coupled transport of di- and tripeptides and peptide-like drugs across cell membranes. The physiological role of PEPT1, one member of this family in mammals, is mainly the uptake of small peptides into intestinal and renal tubular epithelial cells. In *Caenorhabditis elegans* a homologue to mammalian PEPT1 is encoded by the *pep-2* gene, which is expressed in the intestinal cells and a subset of sensory neurons in the head of the animal.

To study the physiological role of the PEP-2 transporter *in vivo*, a *C. elegans pep-2* mutant was constructed. The animals deficient in PEP-2 show a remarkable phenotype with pronounced signs of malnutrition, characterised by a delayed development, less eggs in the uterus, a smaller brood size and a prolonged mean life-span compared to wild-type animals.

We rescued the phenotype by the expression of the wt *pep-2* gene in the mutant. The observed starved phenotype in *pep-2* mutants might be best explained by the reduced intestinal absorption of peptide bound amino acids that are required for protein synthesis and energy metabolism and provides the first direct evidence for the predominant role of the intestinal peptide transporter in amino acid absorption.

Modelling without crystals – What can we infer about the substrate binding site of the proton-coupled peptide transporter (PEPT1)?

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With a few notable exceptions, attempts to crystallise integral membrane proteins have failed due to the difficulties in finding appropriate conditions for proteins that have both hydrophobic and hydrophilic domains. Thus structural information is largely limited to predictions of secondary structure from the amino acid sequence and computer modelling, neither of which can as yet give high resolution detail. Thus alternative approaches are required, and one that we have employed is to look at the substrate binding/transport characteristics of compounds and predict what features the binding site might have. The membrane transport protein that we are interested in is the proton-coupled di/tri-peptide transporter, which has a wide range of natural substrates and is known to transport therapeutically important non-peptides such as □-lactam antibiotics and angiotensin converting enzyme inhibitors.

The initial question that interested us was what makes a di/tri-peptide a substrate, but not an amino acid? While the obvious answer is the peptide bond, studies with 'space mimic' compounds (which have the space filling properties of a dipeptide but no peptide bond) gave the surprising result that the peptide bond was not essential for binding and translocation. Although these space mimics had N and C termini, studies from our laboratory and others have shown that the presence of free amino or carboxyl groups are not a prerequisite for binding or translocation either. This leaves the question of what does distinguish a PepT1 substrate from a non-substrate?

Computer modelling of a large number of PepT1 substrates has allowed the development of a substrate template, whereby potential substrates can be scored according to their predicted binding affinity. From this it is clear that it is a sum of energies derived from a number of substrate-transporter interactions that determine binding affinity, including the N- and C-termini, the peptide bond components and the substrate side-chain groups. Further studies aim to refine this model through the complimentary approaches of novel substrate design and site-directed mutagenesis of the transporter protein.

Why are we interested in this? A large number of promising therapeutic compounds are found to have little or no bioavailability. Compared with most membrane transporters PepT1 has a wide range of potential substrates, and amongst its non-peptide substrates are a range of peptidomimetic therapeutic compounds. The recent finding that a peptide bond is not a prerequisite for transport opens up the possibility of designing prodrugs to be substrates for PepT1, and this has found to be an effective strategy for example with the antiviral drug valacyclovir.

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Experimental studies on the uptake of radiolabelled amino acids in tumor and inflammatory cells

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Aim: The high amino acid metabolism of tumor cells allows tumor imaging with radiolabeled amino acids as ^{11}C-methionine (Met) by positron-emission-tomography (PET). However in recent experimental and clinical studies Met uptake was also found in inflammatory tissue thus leading to false positive results. The aim of the study was to compare [^{18}F]-fluoroethyltyrosine (FET), a new amino acid analogue, with Met to assess their suitability for differentiating between tumor cells and inflammatory cells in vivo and in vitro.

Methods: Popliteal lymph nodes of Balb/c and DBA/2 mice were stimulated either by streptocotocin (STZ), causing chronic lymphadenitis, or by concanavalin A (Con A), causing in acute lymphadenitis. Tumor infiltrated lymph nodes were induced by inoculating cells from a lacZ transfected T-cell mouse lymphoma line into the footpads of syngenic DBA/2 mice. The uptake of Met and FET was determined quantitatively in tumor infiltrated and inflammatory lymph nodes as well as in the lymph nodes of untreated mice. In vivo imaging of tracer uptake in mouse lymph nodes was performed using a high resolution (2.4 mm) small animal PET (MADPET). In vitro the uptake of the amino acids Met and FET was investigated in different cells, such as SW707 human colon carcinoma cells and C6 rat glioma cells, stimulated human lymphocytes and macrophages. About 5×10^5 cells of each cell line were incubated in a buffered medium containing either different concentrations of unlabeled amino acids or Con A (stimulation of lymphocytes) or the transport inhibitors 2-amino-norbornane-carboxylic acid (BCH, L-system), α-(methylamino)-isobutyric acid (MeAIB, A-system) or L-Serin (ASC-system). 0.37 MBq of each amino acid tracer were added and incubated. Uptake was stopped by using ice-cold PBS, cells were washed three times and uptake was analyzed.

Results: In tumor infiltrated lymph nodes uptake of both tracers was higher than in control lymph nodes. Met showed an increased uptake in both lymphadenitis models, whereas FET did not accumulate significantly. Met and FET uptake in tumor infiltrated lymph nodes was also seen in MADPET images, however inflammatory lymph nodes could only be detected in Met images.

The amount of tumor uptake was different in the various cell types investigated. C6 cells showed the highest uptake of all cells investigated and a slightly lower uptake was found in SW707 cells. In Con A stimulated lymphocytes, the uptake of FET was negligible, while Met uptake was significantly higher than in both tumor cell lines. Since BCH reduced the uptake of FET and Met to approximately 10%, FET seems to be also predominantly transported into tumor cells by the L-system.

Conclusion: The results indicate, that FET appears to differentiate between tumor and inflammatory tissue, as a result of the low uptake of FET in inflammatory cells.

Application of radiolabelled amino acids in clinical oncology

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Over the past few years numerous studies have documented the high diagnostic accuracy of positron emission tomography (PET) using the glucose analogue F-18-fluordeoxy-glucose (FDG) for detection and staging of malignant tumors. A significant limitation of FDG-PET, however, is that increased

uptake is not only observed in malignant tumors but also in activated inflammatory cells. Due to the high glucose utilization of the normal brain and the lower protein synthesis in the normal gray matter the radiolabelled amino acid C-11-methionine (MET) gives higher contrast between brain tumors and normal tissue than FDG-PET. Rapid uptake of MET has been documented for several malignant tumors like gliomas, lung cancer, bladder cancer and malignant lymphomas since amino acid transport and protein synthesis are generally increased in malignancies. The application of MET-PET however has been limited by the short half life of the radioactive label C-11 (20 min) in contrast to F-18 (110 min). Amino acid analogous labeled with F-18 like F-18-fluoro-α-methyltyrosine (FMT), F-18-fluoro-ethyltyrosine (FET), F-18-fluoro-phenylanaline, F-18-fluore-proline will allow a more widespread application of amino acid PET in oncology. An other amino acid analogue I-123-iodo-α-methyltyrosine (IMT) is of clinical interest because the radionuclid I-123 allows it applicability for single-photo-emission-computer-tomography (SPECT). The uptake of the amino acid analogues can only be regarded as a measure for the increased amino acid transport in the tumor cells because they are not incorporated into proteins. Clinical data show that radiolabelled amino acids that are only transported into the cells are not inferior to those that enter protein synthesis. This tracers may also help to differentiate tumor lesions from inflammatory lesions when the expression of the transport systems for amino acids in tumor cells and inflammatory cells is different.

Lysinuric protein intolerance: understanding the pathophysiology of a multi-system disorder of dibasic amino acid transport

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Lysinuric protein intolerance (LPI; MIM 222700) is an autosomal recessive disease, mainly found in Finland and Italy. Clinical findings of LPI include: vomiting, diarrhea, failure to thrive, hepatosplenomegaly, osteoporosis, episodes of coma, and mental retardation. A life-threatening lung involvement (alveolar proteinosis) and renal insufficiency were also reported. Metabolic derangement of LPI includes: reduced intestinal absorption of cationic amino acids (lysine, ornithine, arginine, CAA), increased renal excretion of CAA and dysfunction of the urea cycle leading to hyperammonemia and orotic aciduria. Most of the clinical findings cannot be explained by a selective deficiency of amino acid transport, as indeed observed for cystinuria (MIM 220100), a cognate disease of LPI. The molecular basis of LPI resides in an abnormal CAA carrier functioning at the level of basolateral membrane of epithelial cells in the intestine and the kidney. CAA transport is mediated by y + L system, that is exerted by heterodimers consisting of the 4F2 heavy chain (4F2hc) and a light chain represented by either the solute carrier family 7A, member 6 (SLC7A6) or 7 (SLC7A7). After excluding the 4F2hc as the causative gene of LPI, we identified SLC7A7 as the LPI gene and characterized mutations in twenty-five patients from 21 families (16 Italian, 2 Japanese, 1 Moroccan, 1 Greek, and 1 Pakistani; 34 independent alleles) affected by LPI. Thirty-two of the 34 independent alleles (94.1%) were characterized and fourteen mutations were identified. Only five mutations (namely 1625insATCA, W242X, 1425delCTCT, IVS3 + 1G→A, S386R) were identified in more than one independent family. Most mutations are located in the SLC7A7 coding region, except for two splicing mutations. The pathogenesis of some clinical findings of LPI, namely alveolar proteinosis and renal involvement, remains mostly unknown. We are currently investigating the role of SLC7A6 gene in LPI, which, in addition to SLC7A7, is responsible of the y + L activity. In fact, the regulation of the y + L system, exerted by either 4F2hc/SLC7A76 or 4F2hc/SLC7A7, is still unknown. Hypothetically, the activation of 4F2hc/SLC7A76 in all tissues might be the "simple" way to a LPI gene-therapy.

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Increased nitric oxide production in human fetal endothelial cells from pre-eclamptic pregnancies is not accompanied by alterations in L-arginine transport

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Pre-eclampsia (PE) is a potentially life threatening complication of pregnancy and is one of the leading causes of maternal and fetal morbidity and mortality. PE is associated with endothelial cell dysfunction and inadequate placental perfusion. Fetal plasma L-arginine levels are decreased in PE and there is controversy as to whether nitric oxide (NO) production is altered. We have investigated whether the kinetics of L-arginine transport via system y^+ and NO production are altered in fetal umbilical vein endothelial cells (HUVEC) from PE pregnancies. Kinetics of L-arginine transport were similar in HUVEC isolated from normal, preterm and PE pregnancies, however N-ethylmaleimide inhibited transport in normal but not PE HUVEC. Basal and histamine-stimulated NO production was similar in normal and preterm HUVEC, whereas PE increased basal (25 ± 5 vs 5.3×3 pmol/10^8 cell/5 min) and histamine-stimulated (70 ± 12 vs 20 ± 5 pmol/10^8/5 min) NO production. Whole-cell patch clamp measurements revealed similar inward rectifying K^+ currents in normal and PE HUVEC, with resting membrane potentials of -65 ± 4 and -80 ± 18 mV in normal and PE HUVEC, respectively. Increased eNOS activity in PE endothelial cells may serve as a compensatory mechanism to counteract the hypertension observed in PE, however, elevated NO production is apparently not associated with enhanced L-arginine transport.

Antibiotic resistance: Era of the multidrug pump

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Over the past years, concerns have heightened over the escalating numbers of pathogenic microorganisms that are resistant to multiple antibiotics. This phenomenon poses major problems in the treatment of patients with hospital or community-acquired infections caused by bacteria, yeast, fungi and parasitic organisms. Particularly intriguing are the so-called multidrug transporters, which have specificity of compounds with very different chemical structures and cellular targets. This lecture will focus on the molecular properties of the ATP-binding cassette multidrug transporter LmrA in the lactic acid bacterium *Lactococcus lactis*. LmrA is a close homolog of the human multidrug resistance P-glycoprotein, overexpression of which is one of the major causes of resistance of human cancers to chemotherapy. Surprisingly, LmrA can even substitute for P-glycoprotein in human lung fibroblast cells. Recent biochemical and pharmacological studies on LmrA suggest that the protein may operate by a two-cylinder engine mechanism to transport amphiphilic drugs from the inner leaflet of the plasma membrane. This mechanism will be discussed in more detail.

A_{2a} adenosine receptor activation acutely stimulates L-arginine transport and NO production in human endothelial cells

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Adenosine is a potent vasodilator in many vascular beds and modulated tone via elevation of intracellular cAMP and/or release of nitric oxide (NO). We have previously reported that adenosine (ADO) stimulates L-arginine transport and NO production in human cultured umbilical vein endothelial cells (Sobrevia et al., J. Physiol. 499, 135–140, 1997), and here further characterise the signalling cascades. RT-PCR demonstrated that fetal endothelial cell possess mRNA levels for A_{2a}, A_{2b} and A_3-adenosine receptor subtype, whereas negligible levels were detected for the A_1-receptor. Adenosine (10 μM, 2 min) induced increases in L-arginine transport and NO production were Ca^{2+} and cAMP independent and stimulated transport was abolished in cells depolarised with 80 mM K^+. Whole-cell patch clamp experiments revealed that adenosine activated inward K^+ currents, resulting in a membrane hyperpolarization and enhanced influx of the cation substrate L-arginine. Adenosine induced L-arginine transport and NO production were also abolished by inhibitors of tyrosine kinases (genistein), MEK1/2 (PD98059, U0126) but unaffected by inhibitors of PKC (calphosin C) and PI-3 kinase (LY29002). These data suggest that adenosine induces membrane hyperpolarization by activating inward K^+ currents, increasing the driving force for cationic amino acid influx via system y^+.

Addendum

Spermine and amine oxidase induce a cytotoxic effect on multidrug resistant Chinese hamster ovary cells

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The occurrence of resistance to cytotoxic agents in tumor cells is a major obstacle to successful anticancer chemotherapy. Multidrug resistance (MDR) is associated with several phenotypic alterations. Cells with the MDR phenotype display decreased drug accumulation due to overexpression of P-glycoprotein (P-gp), encoded by the *mdr-1* gene, which acts as an energy-dependent pump involved in extrusion of drugs. We studied a new strategy to eliminate MDR cells using an enzyme, bovine serum amine oxidase, capable of forming cytotoxic products, H_2O_2 and aldehyde(s), from polyamines (spermine).

The involvement of both toxic products, formed by the BSAO/spermine enzymatic system, in causing cytotoxicity was investigated in multidrug resistant Chinese hamster ovary cells, CH^RC5, at 37 and 42°C. We observed that hyperthermia, depletion of intracellular glutathione (by L-buthionine sulfoximine) and inhibition of glutathione S-transferase (by ethacrynic acid), sensitized CH^RC5 cells to the cytotoxic effect of spermine enzymatic oxidation products. MDR cells showed no resistance to H_2O_2 and aldehyde(s) relative to their drug-sensitive counterparts, AuxB1 cells, in experimental conditions of: higher temperature, higher spermine concentration and longer incubation time. The inhibition of cellular detoxification systems led to increased cytotoxic effects of spermine enzymatic oxidation products on both MDR and sensitive cell lines.

These results might be of great interest and suggest that toxic oxidation products formed from spermine and amine oxidase could be used in anticancer therapy, mainly against multidrug resistant tumor cells.

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Antibacterial and antiviral effects of new amphiphilic monobactams

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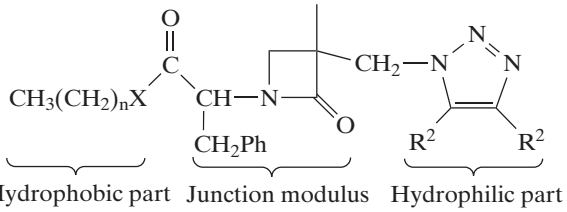
The discovery of Nocardicine A by Aoki et al. and Aztreonam showed that monocyclic β-lactams, collectively known as monobactams, can have antibiotic activity. This activity is poor but compensated by the unique effect they can induce on certain microbial cell membranes.

Our quest for new non-conventional surfactants for various biomedical applications led us to synthesize bioactive compounds with structural similarities to Nocardicins. We present here the preparation and the study of original trimodular biosurfactants of type I:



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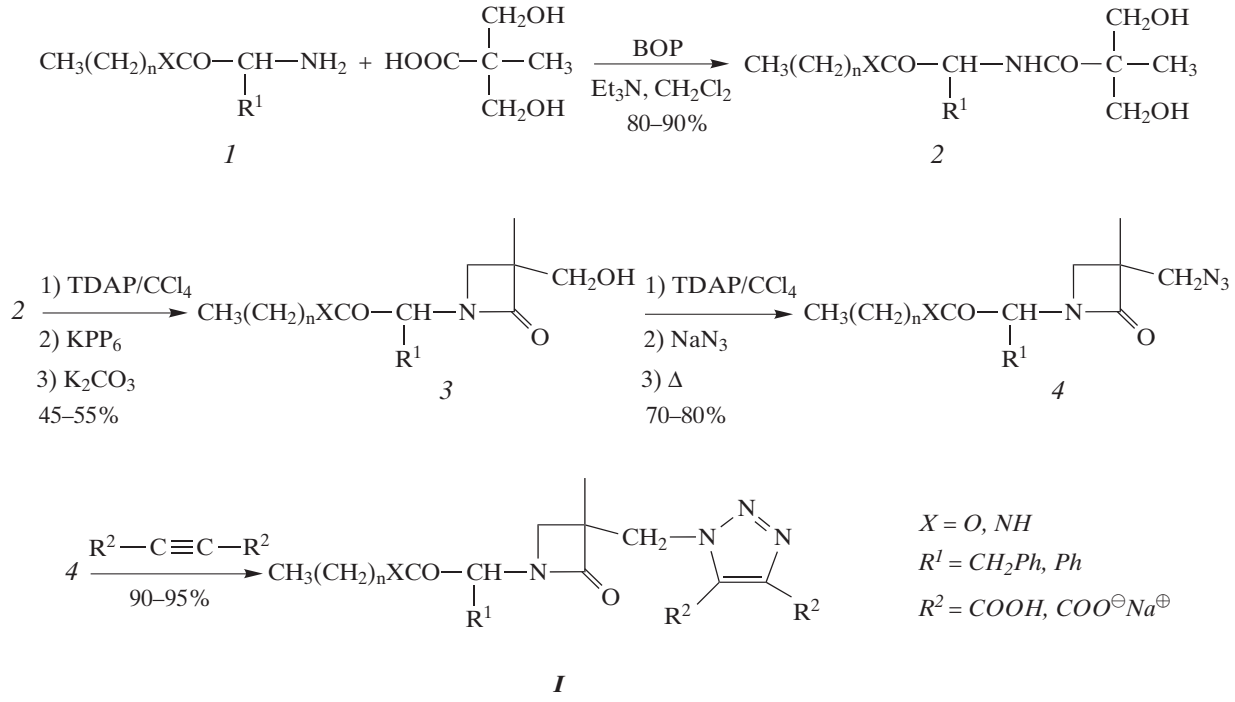
These compounds present a hydrophobic part introduced by an ester or amide linkage with an aminoacid, a junction modulus which corresponds to β-lactam, and a hydrophilic part which contains a triazole, well-known in pharmaceutical industry for its inhibitor effect against β-lactamase.

The compounds are synthesised from 2-hydroxymethyl-2-methyl propionic acid in five steps. Selective activation of one of the primary hydroxyl groups was accomplished by the formation of alkoxy tris(dimethylamino)phosphonium (ATDP) salts 3 from the corresponding diol. Treatment of 3 with excess potassium carbonate in refluxing anhydrous acetone yields the monobactams 4. Activation by ATDP salts followed by treat-



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ment with sodium azide and reflux in toluene gives the azido compound. The reaction with acetylenic derivatives allows to obtain the surfactants.

The compounds show classical surfactant behavior and the evaluation of their biological properties give evidence for their antibacterial and antiviral activity, which corresponds apparently to antiprotease activity.

A prodrug approach to glutathione derivatives with *in vitro* antiparasitic activity

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The potential chemotherapeutic activity of peptides are lost in many cases *in vitro*, due to their inability to cross cell plasma membranes. The recent identification of a series of glutathione diesters with high antiparastic activities *in vitro* against *T.b.brucei* (African Sleeping Sickness) lead us to investigate the determinants associated with their activity. A QSAR study on some twenty-five diester derivatives against T.b.brucei and T.b. rhodesiense lead us to conclude that the mechanism of action of these compounds is related to membrane penetration and hydrolysis, controlled by hydrophobicity and steric factors. A HPLC and sensor study have confirmed the de-esterified diacid as the active agent of these prodrugs.

Dietary taurine prevents oxidative stress and morphological alterations in the retina of diabetic rat

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Diabetes mellitus can cause various complications including retinopathy, which is the earliest and most common complications of diabetes mellitus, affecting 90% of diabetics and progressing to blindness in about 5%. Considerable evidence implicates oxidative stress in the pathogenesis of diabetic retinopathy. In fact, hyperglycemia generates reactive oxygen species and free radical defense is reduced in diabetic patients. Thus, the prevention of oxidative stress may have important implications for pharmacological attempts to prevent diabetic retinopathy. At this regard, it has been found that taurine, a semi essential amino acid with antioxidant activity, is decreased both in type 1 and type 2 diabetes mellitus. Moreover, taurine seems to have a peculiar role of taurine in terms of cellular physiology and pathophysiology of the retina. Among others, taurine is thought to produce important physiological effects through osmoregulation, calcium modulation and antioxidant effects.

Therefore, we examined the effect of dietary chronic (4 months) taurine (2% and 5%) supplementation in diabetic rats in comparison with vitamin E (200 and 500 UI). Dietary taurine supplementation, for 4 months, does not influence conjugated dienes (CD), lipid peroxides (LP) and Na/K ATPase activity in the retina of non diabetic rats. Using rats streptotozocin (STZ) induced diabetes of 4- month duration, we found that CD, LP are significantly increased and they remained elevated for 4 months. While, the Na/K ATPase is significantly decreased during the whole experimental time (4 months). Moreover, an inverse correlation has been found among the CD and LP and ATPase activity. In the retina of STZ rats, these biochemical alterations are accomplished with marked profound morphological changes.

In STZ rats, taurine enriched diets decrease the lipid peroxidation and preserve the ATPase activity, being 5% taurine more effective than 2% diets. The morphological examination reveals that in rats feed with 5% taurine no proliferative changes are present. Moreover, the beneficial effects of taurine are more marked than of those of vitamin E.

These results and previous findings encourage new investigations to evaluate the efficacy of taurine as an adjunctive agent

to ameliorate the course of diabetic complications considering the low cost and the low toxicity of this amino acid and the importance of disease.

Connective tissue antigens in breast cancer

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Bone and bone marrow are important sites of metastasis formation in breast cancer; so, we studied the level of bone sialoprotein (BSN) and fibronectin (FN), two key connective tissue antigens, in patients with metastatic breast carcinoma. Our data reveled that BSN have a statistically significant association with bone metastases in that disease. FN level was also significantly changed in metastatic breast carcinoma when compared to the non metastatic cases.

Study of temperature induced structural transitions in proteins

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In the work the temperature dependencies of dielectric parameters of human serum albumin (HSA) and fibrinogen solutions (0.15 M NaCl, pH 7.2) were obtained in the temperature interval 5–50 C degrees. The measurements of the dielectric parameters were carried out at the frequency of 9.2 HHz, i.e. in the range of free water molecules dispersion. In contrast to dependencies for poor solvent, temperature dependencies of dielectric parameters for protein solutions are of non-monotonous character; they have a number of peculiarities in the temperature ranges of 8–10, 22–24 and 34–36 C degrees. This fact means that at these temperatures redistribution of free and bound water in protein-water system occurs due to structural changes in protein molecules. The dependencies of hydration of HSA and fibrinogen on temperature were obtained as well.

In the work the mechanism of temperature changes of spatial organisation of protein molecules was proposed. Perhaps, this mechanism is responsible for maintenance of thermal stability of the functionally active conformation of native proteins. As peculiarities on temperature dependencies of dielectric parameters of solutions of globular (HSA) and fibrillar (fibrinogen) proteins were in the same temperature regions, one may to assume that the mechanism of proteins thermal stabilisation in physiological temperatures interval has a general character.

Isoforms of the N-terminal POMC fragment (N-POMC) secreted by AtT-20 pituitary tumor cells and their effect on lactotrophs

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N-POMC was purified from conditioned medium of AtT20 cells using a sequence of concentration, fractionation by ion exchange, RP-HPLC and gel-filtration. Twenty isoforms of N-POMC, for both 11 and 13 kDa, were identified by means of mass spectrometry and N-terminal sequencing. These isoforms are assumed to be POMC1-74 or POMC1-95 with heterogeneous glycosylation.

The N-POMC isoforms were tested on prolactin (PRL) gene expression and lactotroph mitosis in pituitary cell aggregate cultures. PRL mRNA content was quantified by means of real time RT-PCR. Three 11 kDa N-POMC fractions enhanced PRL mRNA levels by 33–36%, while all other isoforms were inactive. This effect was abolished by immunoneutralization with N-POMC monoclonal antibody. Only one fraction stimulated lactotroph proliferation (38.2 ± 7.5%) as assessed by BrdU incorporation in PRL–immunoreactive cells. Several (but not all) 13 kDa N-POMC fractions stimulated PRL mRNA level and lactotroph mitosis. On the other hand, all 11 and 13 kDa isoforms activated the MC-3 and MC-5 receptor in cell lines in which these receptors were transfected. Thus, AtT20 cells produce various N-POMC isoforms, only a part of which display an effect on PRL mRNA expression. Even fewer isoforms affect lactotroph proliferation. Since all isoforms activate the MC-3 and MC-5 receptor, it is suggested that the effect of the few isoforms on lactotrophs is mediated by (a) different receptor(s).

The effect of hyperforin on amino acid neurotransmitter release from mouse cortical slices

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Extracts of St. John's Wort (*Hypericum perforatum L.*) are widely prescribed for the treatment of mild to moderate depression and the putative antidepressant constituent is probably hyperforin. In this study the effect of hyperforin was investigated on the release of neurotransmitter amino acids.

Coronal cortical slices (400 μm) were cut and perfused with gassed (95% O_2, 5% CO_2) aCSF at 37°C. Two-minute samples of perfusate were collected and aspartate and glutamate were assayed by HPLC. Potassium- and veratridine-stimulated release was elicited by administering 2 pulses of K^+ (60 mM) or veratridine (20 μM) 30 minutes apart.

In control experiments the second K^+ pulse elicited glutamate release which was 80% of the first pulse. Hyperforin (5 μM) perfused for 30 minutes prior to, and during, the second K^+ pulse significantly increased glutamate release to 170% ($P < 0.001$, $n = 6–8$). Release elicited by the second veratridine pulse was 70% of the first pulse for both glutamate and aspartate. Hyperforin (5 μM) increased this release to the second pulse to 160% and 130% respectively ($P < 0.001$, $n = 6–8$). When perfused on its own for 30 minutes, hyperforin (5 μM) increased the basal release of glutamate ($P < 0.001$, $n = 4–5$).

In conclusion, the increase in the release of neurotransmitter amino acids observed following hyperforin is possibly mediated through a facilitatory action on voltage-operated Ca^{2+} or Na^+ channels.

Effects of glucagon like peptide-1 on γ-aminobutyric acid and serotonin metabolism in synaptosomal fractions from rat hypothalamus and brain stem

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Glucagon-like peptide-1 (7–36) amide (GLP1) is the main product of the glucagons gene expression in intestinal L cells into the cirulation in response to the ingestion of food and is the most potent stimulator of glucose-induced insulin secretion. GLP1 receptors have also been detected in discrete areas of rat brain and intracerebroventricular GLP1 has been shown to inhibit feeding in fasted rats. In this study HPLC techniques were employed to evaluate the effects of GLP1 on Serotonin (5-HT) and γ aminobutyric acid (GABA) metabolism in rat brain. GLP1 (0.5 μM) produced a significant decrease in levels of 5-HT by 20% after 15 minutes of incubation with combined hypothalamus and brain sterr. synaptosomes. Levels of 5-hydroxyindolacetic acid (5-HIAA), the principal metabolite of 5-HT, and tryptophan the amino acid precursor of 5-HT, were also decreased significantly by 21% and 37% respectively. GABA and its amino acid precursor glutamic acid were both measured at the same conditions as above, but a precolumn derivatization HPLC technique was used. The increase in levels of GABA (14%) and Glu (6%) by GLP1 was not significant.

The results suggest that decreased synaptosomal levels of 5-HT and 5-HIAA caused by GLP1 are due to diminished availability of typtophan by the peptide.

Effect of Spermine on liver oxidative stress during acute iron overload in rats

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In experimental model of iron overload we obtained the following results: the concentration of carbonyl groups tended to increase, while MDA level significantly increased after $FeSO_4$ treatment (1.66 ± 0.25 vs control 1.51 ± 0.50 μmol/mg prot.) and (2.13 ± 0.5 vs control 1.3 ± 0.3 nmol/mg protein $p < 0.01$) respectively. It was associated with significantly increased iron content (0.89 ± 0.23 μg/mg prot. vs control 0.49 ± 0.17 $p < 0.001$). It is clear that oxidative stress occurs in experimental iron overload, if sufficiently high levels of iron within hepatocytes are achieved. In group treated with $FeSO_4$ and spermine, iron content was significantly decreased (0.36 ± 0.07 $p < 0.01$ compared with Fe treated only) and carbonyl group content tended to be lower in comparison to $FeSO_4$ treated only (1.58 ± 0.24), but MDA level didn't change (2.31 ± 0.72). In addition, treatment with spermine alone resulted in increase of MDA level (2.74 ± 0.7 vs control $p < 0.01$), iron content didn't change (0.59 ± 0.29), but carbonyl groups were decreased (0.99 ± 0.28 vs control $p < 0.05$). $FeSO_4$ treatment increased GSH level (126.38 ± 34.11 nmol/mg prot. vs control 88 ± 22.77; $p < 0.05$) while in combination with spermine this increase was more profound (235.48 ± 42.7; $p < 0.001$ vs control, $p < 0.001$ vs $FeSO_4$). Spermine alone produced similar increase of GSH level (127.4 ± 34.11, $p < 0.05$ vs control; $p > 0.05$ vs $FeSO_4$).

Proteomics and the chemical synthesis of proteins

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The results emanating from the Human Genome Programme have required a reappraisal of protein science and have led to the rapid upsurge in interest in the area of proteomics. This sudden re-emergence of protein science, in fact, was predictable and should not have been surprising.

Recent experience of protecting group design with respect to lysine and aspartic acid will be discussed together with aspects of chemical synthesis of small proteins of biological significance and in the context of chemical synthesis methodology making contributions to the general field of proteomics.

Mechanism of taurine transport inhibition by Cyclosporin A

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Using a cell line permanently expressing the mouse taurine transporter (mTauT) as a fusion protein, we investigated the underlying mechanism by which the immunosuppressive drug cyclosporin A (CsA) inhibits taurine transport. CsA inhibited the recombinantly expressed mTauT function both in dose and time dependent manner. The inhibitory effect of CsA was reversible. Thus, washing out the CsA resulted in almost complete recovery of taurine uptake. To obtain further insight, we examined the surface abundance of the mTauT as a function of CsA treatment using a surface-labeling assay. Our results demonstrated that CsA treatment altered the surface expression of the mTauT without significantly altering its total expression level, and the reduction in the cell surface expression paralleled the decrease in taurine uptake. Upon removal of CsA, the virtual recovery in taurine uptake was due to the concomitant increase in the number of taurine transporters on the cell surface. Taken together, our results suggest that CsA induced inhibition of taurine uptake was either due to the impaired targeting of the taurine transporters to the cell surface or due to the removal of the transporters from the cell surface.

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Altered polyamine metabolism influences diminshed neural inhibition during 2-butoxyetanol toxicity

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Polyamines are neuromodulators in a number of physiological and pathological conditions in CNS. Since application of ethylene glycols causes hypoactivity and lethargy of experimental animals, depression of CNS and various neurological symptoms, the aim of this study was to examine the effects of 2-butoxyetanol on polyamine and GABA catabolism, taking in account an alternative pathway of GABA synthesis from putrescine.

Methods: Male Wister rats were allocated into three groups: first treated by BE (100 mg/kg – 10 days), second

applicated BE (500 mg/kg – 10 days) and the third – control. Enzyme activities were determined spectrophotometrically in brain homogenate. Results: Polyamine oxidase activity decreased significantly ($p < 0.001$) in both experimental groups (2.66 ± 0.12; 2.79 ± 0.04; U/mg prot) in a dose dependent manner, compared to control values (3.3 ± 0.03). Lower dose of BE didn't induce any significant change in diamine oxidase activity, while higher dose led to significant ($p < 0.05$) decrease (2.56 ± 0.13 vs control 2.83 ± 0.02). GABA-transaminase activity increased significantly ($p < 0.005$; $p < 0.001$) and dose dependently upon BE treatment (0.71 ± 0.04; 1.24 ± 0.07) in relation to control (0.51 ± 0.02).

Conclusion: Diminished polyamine catabolism points out the possibility of polyamine involvement in modulation of CNS inhibition during BE influence, as a part of synchronized effects of a number of mechanisms resulting in BE-induced CNS toxicity.