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Rapid determination of elastin crosslink amino acids in tissue hydrolysates by isocratic HPLC and precolumn derivatization

Desmosine (Des) and Isodesmosine (Ide) are the major specific crosslink amino acids in mature elastin. The content of Des and Ide is considered as an index of elastin amount in tissues, and the presence of these two compounds in biological fluids is a marker of elastin breakdown. The low concentrations of Des and Ide are highly unbalanced compared with total amino acid composition. Therefore, there is a need for sensitive and accurate methodologies in order to analyse elastin crosslink content in tissues or in purified elastin.

We report here a rapid and sensitive isocratic high performance liquid chromatographic method for the single and specific determination of low concentrations of desmosine (Des) and isodesmosine (Ide). Samples of isolated elastin or whole tissues were hydrolysed in 6N HCl, at 105°C for 18 hours and the hydrolysates were prefractionated on CF1-cellulose. This prefractionation step was found to be efficient to remove most of the non crosslink amino acids. Des, Ide and γ-glutamyl-glutamic acid (γ-glu-glu) as internal standard, were dansylated, according to an optimized dansylation procedure. The reaction was stopped after 15 min with ethylamine. Then, Des, Ide and internal standard derivatives were extracted from reaction mixture by ethylacetate. The ethylacetate extraction procedure eliminated unwanted derivatives and derivatization side-products. The chromatography was performed on a Lichrospher 100-NH_2 column, using methanol-water as mobile phase containing acetic acid and 0.25 M sodium acetate, final pH 6.5, and effluent was monitored by fluorescence detection (340–510 nm). A 30 minutes isocratic run allowed a clear separation of Des, Ide and internal standard. The overall reproducibilities of the complete procedure, including CF1 prefractionation, were 5.9% for Des and 5.0% for Ide (coefficient of variation). The limits of detection were 2.2 pmol and 2.5 pmol, respectively. The method was successfully applied to the determination of Des and Ide in normal pig aortas and could be further applied to assay of elastase activities.

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Morphometric and biochemical analysis of homocysteine-induced alterations of vascular elastin in a dietary experimental model of hyperhomocysteinemia in the mini-pig. Evidence for a preventive effect of Captopril-Hydrochlorothiazide association

Homocysteine is considered as an independent risk factor of atherosclerosis, but mechanisms of its toxicity remain unknown. We report here a dietary model of hyperhomocysteinemia in the mini-pig, using a rich methionine diet overloading the transsulfuration pathway. Preventive effects of a treatment by a Captopril-Hydrochlorothiazide association (Cp), were tested.

32 mini-pigs randomized into 4 equal groups, received different proteinic diets and treatments for 4 months, and were then sacrified. The control (C) diet contained 21% of vegetal proteins (1.28 g methionine/day), and the methionine (M) diet 30% of calcium caseinate (3.45 g methionine/day). The treated groups (C + Cp) and (M + Cp) received daily oral doses of 25 mg of Captopril and 12.5 mg of Hydrochlorothiazide (Htz).

Blood samples were taken monthly: biological parameters did not vary significantly, nor inside a given group, nor between the different groups, proving that all the animals remained healthy during the protocol. Only 2 mini-pigs of the M group died, one with an acute cerebral stroke, the other with a pulmonary embolism, attesting the vascular impact of the M diet. Homocysteinemia increased significantly from the 1st month: 9.6 ± 4.1 μmoles/l in the (M) + (M + Cp) groups ($n = 16$) and 5.6 ± 1.1 μmol/l in the (C) + (Cp) groups ($n = 16$) ($p < 0.05$). This hyperhomocysteinemia, stable all along the protocol, was not modified by the Captopril + Htz association. The inhibition of plasma angiotensin converting enzyme (ACE) activity reached progressively 75% in the treated groups.

The impact of hyperhomocysteinemia on the arterial wall elastin was studied. The elastin contain of the sub-renal aorta (mg/100 mg dry tissue), decrease significantly in the M group (16.7 ± 1.5) versus the C group (18.5 ± 2.1) ($p < 0.05$) and the C + Cp group (19.0 ± 1.6). In the M + Cp group the level (17.8 ± 2.1) was not significantly different of that of the C group. The crosslink aminoacids, desmosine and isodesmosine, were evaluated by HPLC. Their rates were not significantly different between the different groups, that as to say that elastin kept

a normal reticulation during the different diets or treatments. The hypothesis of an increased elastase activity seemed more likely than a decreased elastin synthesis, due to the short time of this protocol compared with the low turn-over of elastin. Histological study of abdominal aorta showed hypertrophic endothelial cells, prominent fragmentation of elastic laminae, hypertrophy and reorientation of medial cells, and communicating pathways between muscular laminae. In treated M + Cp group the hyperhomocysteinemia-induced alterations of elastic laminae were moderate, laminae stacking-up was preserved, and communicating pathways between muscular laminae were rarely noted.

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Rat kidney glutathione levels and its related enzymes in renal failure

Several *in vitro* studies have shown the protective effect of glutathione (GSH) on oxidant injury to the proximal renal tubules. Still, the role of GSH in *in vivo* models of renal injury, in which reactive oxygen metabolites have been postulated to play an important role remains controversial.

To gain greater insight of the role of glutathione (GSH) in acute renal failure (ARF), we studied the changes in GSH levels and its associated enzymes in kidney tissue of rats undergoing ARF produced by i.m. injection of 50% glycerol (10 ml/kg b.w.) and by a single i.p. injection of cis-Platinum (cis-Pt, 5 mg/kg b.w.). In both models of ARF, kidney damage was characterized by increases in relative kidney weight and BUN levels. The changes in the parameters tested, presented in this report, occurred in the kidneys within 24 and 72 hours after glycerol treatment and 7 days after injection of cis-Pt.

The sustained and pronounced elevation of renal GSH concentration in response to glycerol and cis-Pt treatment (173% and 188% of the control values, respectively) was observed. The elevation in the kidney GSH level was not accompanied by an increase in the activities of glutathione reductase and γ-glutamylcysteine synthetase, enzymes involved in production of GSH. Indeed, the activity of synthetase was substantially inhibited in response to glycerol and cis-Pt treatment, while glutathione reductase activity decreased or remained unchanged, respectively. Moreover, the activity of γ-glutamyltranspeptidase, which catalyzes the initial cleavage of the GSH in the γ-glutamyl cycle, was increased in both types of ARF. The activity of glutathione-peroxidase, which utilizes GSH in the course of inactivation of free radicals, was decreased in glycerol-treated animals, and hence could contribute to the elevation of the renal GSH content. In cis-Pt-treated animals glutathione peroxidase activity was unchanged. Glutathione S-transferase activity was significantly elevated in cis-Pt mediated ARF, but markedly decreased in glycerol-treated rats. Our *in vitro* studies demonstrated that cis-Pt does not directly interact with GSH or enzymes tested.

It is proposed that the elevation of renal GSH level may be caused by an enhanced uptake of GSH and/or cystine-glutamate dipeptide, which, in turn, is probably the result of drug-induced changes in renal membrane transport system.

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Does the free amino acid pool reflect embryogenic capacity of callus tissues and differentiation of young seedlings of *Picea abies*?

In *Picea abies* (L.) Karst. the free amino acid pool of embryogenic and non-embryogenic callus lines has been compared with that of different parts of germinating seeds (roots, hypocotyls, cotyledons and megagametophytes). Amino acids were purified by cation exchange resin and analysed by HPLC using Li-eluent with a pH gradient. The amino acids were detected after reaction with ninhydrin (130°C) at 500 nm.

The amino acid composition was rather similar for roots and hypocotyls: having glutamine as the predominant amino acid at different stages of germination. Glutamine together with arginine, was the most abundant in cotyledons which were obtaining mobilized storage nitrogen via their tips from the megagametophyte tissue (primary endosperm). This organ contained arginine as the predominant amino acid, along with other members of the glutamate family (glutamine, glutamate, GABA and proline) in relatively equal proportions.

During germination in the cotyledons, the amount of arginine increased and that of glutamine decreased. These two amino acids may play a central role in the amino acid metabolism of developing needles and also serve as translocation forms of organic nitrogen. The level of asparagine was much lower and seemed to increase during germination in each part of the seedling.

In organic nitrogen auxotrophic and heterotrophic callus tissues, alanine was the most abundant free amino acid. High levels of asparagine and glutamine were characteristic of embryogenic amino acid heterotrophic callus lines of embryo and megagametophyte origin, but this trend was not observed in the amino acid autotrophic callus line of embryo origin. The green callus lines were not embryogenic, and the level of asparagine was lower than in white embryogenic callus lines, whereas it was about the same as in white non-embryogenic lines. The amount of aromatic amino acids (phenylalanine and tyrosine) was higher in non-embryogenic callus lines than in embryogenic ones.

Arginine and glutamine served as good nitrogen sources for the growth of several amino acid heterotrophic callus lines of *P. abies*. It was possible to induce somatic embryogenesis in some callus lines with amino acid supplementation; asparagine was especially effective. It can thus be concluded that the weakly organized structure of different callus lines is reflected in the free amino acid pool, but physiological characteristics (amino acid autotrophy – heterotrophy; embryogenic – non-embryogenic), origin of the tissue (embryo or megagametophyte), and cytodifferentiation (chloroplasts or amyloplasts) must be taken into consideration when results are interpreted. Young seedlings have a rather dissimilar nitrogen metabolism and a dissimilar free amino acid pool compared to that of callus lines, apparently due to the central role of the protein rich megagametophyte as the source of translocated amino acids in the seeds.

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Sucrase-isomaltase/maltase-glucoamylase complex of ostrich jejunum

The intestinal brush-border glycosidases of most higher animals constitute heterodimeric complexes such as the maltase-

glucoamylase (MG), sucrase-isomaltase (SI) and lactasephlorizin hydrolase (LPH) complexes. These enzymes are anchored to the brush-border membrane on the luminal side by hydrophobic sequences found in one of the two subunits. The final digestion of carbohydrates is accomplished by the three glycosidase complexes, each subunit of the three complexes possessing a specific hydrolytic activity, except maltose hydrolysis which is present on the maltase as well as sucrase and isomaltase subunits. Avian species lack the LPH complex due to the lack of lactose in their diets.

In a study on the distribution of the glycosidase complexes in ostrich intestinal tracts it was found that the LPH complex was lacking as expected. The MG and SI complexes were found in high concentrations in the jejunal area with moderate levels in the ileum, but low levels in the duodenum.

Jejunal mucosa was used to purify the glycosidase complexes of the ostrich from semipure brush-border membranes. The glycosidase complexes were solubilised from the membranes using papain digestion. Solubilised glycosidases were further purified using Toyopearl-Q650C and phenyl-Sepharose CL-4B chromatography. Both the MG and SI complexes coeluted during all purification procedures.

Kinetic characterisation was performed on the purified complexes, including pH and temperature optima, as well as K_m, k_{cat} and k_{cat}/K_m determinations. Inhibition by Tris, deoxynojirimycin, castanospermine and metal ions was tested. Heat inactivation studies were also performed to establish whether the ostrich jejunum possessed two separate glycosidase complexes rather than a single complex with four hydrolytic activities.

Native gradient PAGE gave a relative molecular weight of 420 000 for the ostrich glycosidase complex, while SDS-PAGE, under reducing conditions, revealed the presence of three components corresponding to relative molecular weights of 160 000, 120 000 and 110 000, similar to those expected for the SI complex.

The existence of a single glycosidase complex in the ostrich intestinal tract with four glycolytic activities would be a unique discovery, as all other species to date have been shown to have two separate glycosidase complexes.

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Muscular exercise and arginine glutamate acute oral load in sportsman: I-Plasma ammonia, lactate, and amino acids

Following muscular exercise, there is simultaneous increase of both synthesis and breakdown of skeletal muscle proteins. The nitrogen balance may be negative in the first days of training in spite of an adequate intake of dietary protein. Intense exercise can induce a metabolic acidosis by lactate production, and an enhanced ammoniogenesis consecutive to the oxydation of amino acids (AA).

For top level sportsmen, who exercise each day, it could be interesting to protect the muscle from the protein breakdown. The purpose of the study was to examine, during intense and prolonged cycling, the effect of an oral arginine glutamate salt intake (AGs) on plasma ammonia and lactate, and on the modification of the pattern of amino acids in the plasma in top-level endurance cyclist.

Three healthy top level male cyclists were selected for this study (18, 21 and 22 years old). A double blind design was used with each subject and the order of the intakes with AGs or placebo (Pl) was presented in a cross-over configuration. Each subject was his own control.

Blood samples (10 ml) were taken immediately before the treatment and then at regular intervals of 30 minutes during 90 min and ten hours after the end of exercise.

At rest, AGs intake did not alter the concentration of any parameters. During exercise, arm venous blood lactate greatly decreased from the start of the warming-up and during all the exercise (50% of the pre-exercise value (PEV) $p < 0.05$). The decrease of blood lactate was lesser and occured sooner after AGs intake than after Pl (40% PEV, 60 min with Pl vs 50%, 30 min with AGs).

In opposite, during exercise, the plasma ammonia increased about 3 fold with Pl and 1.5 fold with AGs ($p < 0.01$).

At rest, AGs intake did not induce significant changes in the plasma aminogram except for Asp, Orn, Glu and Arg. At rest, Arg increased 30 min after AGs intake (230%) and then decreased by 25 to 75% of the maximum value within 2 h.

During exercise, for Arg the curve was the same as at rest but it increased later. AGs induced a greater increase of Orn during exercise than at rest. BCAA decreased slowly under AGs. But during exercise, the decrease was greater from 25% to about 35% under Pl or AGs intake. After exercise recovery was slow and still incomplete 10 h after.

In conclusion, this study provides evidence that severe exercise disturbs slightly the profile of plasma amino acids. But the highly trained cyclists are able to sustain a high level of energy out put without any increase in net protein catabolism. The AGs supplementation improves the detoxication of ammonia via the glutamine synthesis, stimulates alanine synthesis and perhaps neoglucogenesis by the alanine and glutamine pathways.

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Muscular exercise and arginine glutamate acute oral load in sportsman: II-Plasma insulin, cortisol, and growth hormone

During exercise, large modifications of hormonal secretions occured, accompanying the substrate and O_2 utilization. The ingestion of different supplementations, supposed with ergogenic effects, could modify the hormonal secretions and adds or counteracts the benefits effects of endurance exercise.

The purpose of this study was to follow the effect of an amino acid supplementation in plasma insulin, cortisol and hGH levels during 1 h intense exercise.

Three healthy male volunteers were tested in three sessions, at rest with arginine glutamate salt (AGs) (20 g) and during exercise with AGs or placebo (Pl) (4 g Aspartam^R). Subjects were instructed to warm-up on a cycle simulator for 30 min at 30% VO_2 max. Blood samples were withdrawn at 0, 30, 60 min during the exercise period and 30, 60 min after exercise. One week later, the experiment was repeated with the subjects receiving Pl or AGs. The Kruskal and Wallis nonparametric H test was used for the comparison between Pl and AGs conditions with subjects.

At rest, with AGs intake, plasma insulin and cortisol did not change. Plasma hGH increased but the increase was significant only at 120 min after AGs intake ($p < 0.01$).

During exercise, insulinemia decreased in the first minutes of the warming-up. ($p > 0.10$) With AGs intake, the decrease of insulinemia became significant ($p < 0.05$). Plasma cortisol level increased non significantly during and after exercise ($p > 0.10$).

Plasma hGH increased ($p < 0.001$) during intense exercise only and peaked up at 30 min with Pl and at 60 min with AGs. This increase in plasma hGH was lower (50%) with AGs than with Pl.

These results showed that during intense exercise, with AGs ingestion, plasma insulin decreased and magnitude in plasma variation of hGH was lower. These effects suggest an interference between Arg and Glu or an action mediated by their glycogenic effect.

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The excretion rates of γ-carboxyglutamic acid in urine correlate with the resting metabolic rates in variously sized mammals

In variously sized mammalian species the whole body turnover rates of protein per unit body weight are highly correlated with the energy turnover rates (resting metabolic rates, RMR) per unit body weight as been shown using stable isotope techniques. We have found in variously sized mammalian species that the whole body degradation rates of tRNA, rRNA and mRNA per unit body weight are also highly correlated with the RMR per unit body weight. From these results we have hypothesized that there is a relationship between the degradation rates of RNAs and proteins and the energy turnover, i.e. the oxygen consumption, possibly via damaging effects of oxygen radicals.

In the present study we investigated in mammals of various sizes at metabolic equilibrium if the urinary excretion rates of γ-carboxyglutamic acid (Gla) per unit body weight correlate with the metabolic activity (RMR) per unit body weight. Gla is formed posttranslationally by carboxylation of specific glutamic acid residues in various proteins (prothrombin; coagulation factors VII, IX, X; plasma proteins C, S, Z; osteocalcin; matrix Gla-protein). Urinary Gla can be used as a marker for the whole body degradation of the proteins from which it originates because it is quantitatively excreted, as has been shown in rats.

In the present study urinary Gla was determined via HPLC after derivatization of Gla with o-phthalaldehyde. The resting metabolic rates (RMR) were calculated on the basis of body weights using an empirical formula: RMR (kJ/kg/d) $\triangleq 240 \times$ kg body weight$^{0.74}$/kg body weight.

We have found in human adults (n = 15; 70 kg), preterm infants (n = 18; 2.0 kg), sheep (n = 7; 64.9 kg), goats (n = 6; 36.5 kg) and rats (n = 22; 335 g) a high correlation ($r = 0.971$; $p < 0.01$) between the mean excretion rates of Gla (μmol/kg/d) and the corresponding values of the RMR (kJ/kg/d). Excretion rates of Gla: 0.92 ± 0.21 (human adults), 2.1 ± 0.76 (preterm infants), 0.92 ± 0.18 (sheep), 1.6 ± 0.2 (goats), 4.5 ± 0.8 (rats); corresponding values of RMR: 80, 201, 65, 94, 320.

From these results we conclude that in variously sized mammals there seems to be a relationship between the degradation rates of Gla containing proteins and the energy turnover, i.e. the oxygen consumption, which would be in accordance with our above mentioned general hypothesis.

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The degradation rates of muscle protein determined via urinary m^{3}His correlate with the resting metabolic rates in human adults, preterm infants and rats

3-Methylhistidine (m^{3}His) is formed posttranslationally by methylation of one histidine residue per actin polypeptide chain and one histidine residue per myosin heavy chain of white fast-twitch muscle fibers. After degradation of the actin and myosin molecules m^{3}His is excreted in some species (e.g. man and rat) virtually quantitatively in urine. The excreted amount of m^{3}His can be used as an indicator of the degradation of skeletal muscle because urinary m^{3}His stems for the most part from this protein source. In the present study we investigated in human adults, preterm infants and rats of different weights if the degradation rates of muscle protein (determined via m^{3}His) per unit of muscle mass correlate with the metabolic activity (resting metabolic rate, RMR) per unit body weight.

Previously, it has been shown by stable isotope techniques that the whole body protein turnover rates in variously sized mammals are highly correlated with the energy turnover rates (RMR). We have found in variously sized mammals that the whole body degradation rates of tRNA, rRNA and mRNA per unit body weight are also highly correlated with the energy turnover rates (RMR) per unit body weight. From these results we have hypothesized that there is a relationship between the degradation rates of proteins and RNAs and the energy turnover, i.e. the oxygen consumption, possibly via damaging effects of oxygen radicals.

In the present study m^{3}His in urine samples of human adults (n = 6, 66 kg), preterm infants (n = 42, 1712 g), rats (n = 22, 78 g; n = 22, 352 g) was determined by classical amino acid analysis (cation exchange resin, derivatization of amino acids with ninhydrin). Determinations of muscle protein degradation via urinary m^{3}His were based on the following assumptions: 1 g degraded muscle protein corresponds to a urinary excretion of 4.2 μmol m^{3}His in human adults, 3.2 μmol m^{3}His in preterm infants and 3.85 μmol m^{3}His in rats; average relative muscle mass in % of body weight corresponds to 45% in human adults, 21% in preterm infants, 45% in adult rats and 34% in young rats. The resting metabolic rates (RMR) were calculated on the basis of body weights using an empirical formula: RMR (kJ/kg/d) $\triangleq 240 \times$ kg body weight$^{0.74}$/kg body weight.

Based on m^{3}His excretion rates we have determined the following relative degradation rates of muscle protein (g/kg muscle mass/day): 1.1 ± 0.1 (human adults), 3.3 ± 0.8 (preterm infants), 4.3 ± 0.8 (adult rats) and 6.4 ± 1.1 (young rats). We have found a high correlation ($r = 0.992$, $p < 0.01$) between these degradation rates of muscle protein and the corresponding values of the RMR (kJ/kg/d): 80, 202, 320 and 466.

We conclude that there seems to be in variously sized mammals a relationship between the degradation rates of muscle protein and the energy turnover, i.e. the oxygen consumption, which would be in accordance with our above mentioned general hypothesis.

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A scheme for the interpretation of amino acid profiles

A scheme for the interpretation of amino acid profiles of biological fluids is proposed. The use of relative concentrations and of the 570/440 nm ratio for the identification of amino acids seem to be the most crucial steps of this scheme. The aim of this scheme is to incorporate the vast biochemical and physiological knowledge of amino acids in healthy subjects and in patients to interpret amino acid profiles. Such strategy would allow the development, on a computer, of a suitable algorithm for interpretation and presentation of results. An example of results obtained from analysis of biological fluids in a patient suffering from lysinuric protein intolerance is shown.

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An intra- and interlaboratory quality control for assay of amino acids in biological fluids: Fourteen years of the french experience

The functioning of an external quality control scheme for amino acids set up in 1978 is described. Two measurements were made each month by participating laboratories on a control plasma sample provided by the quality control centre, using freeze-dried samples from 1978 to 1989 and liquid samples since 1990. The validity of the liquid sample control is demonstrated. Every three months, overall results and those of individual laboratories were analyzed statistically and show for each of the 29 amino acids assayed: the mean, the median, the standard deviation, the coefficient of variation, the range, the number of values, as well as a histogramm of the distribution. In addition, two "blind" samples were sent to the laboratories each year and received the same statistical analysis. Results are shown for: 1978, 1984 and 1992. The progressive improvement of results is spectacular. In 1992. the coefficients of variation for all participants ranged from 7.2% for glycine to 24.8% for aspartate + asparagine. No correlation could be established between the methodology used and the coefficients of variation, the great majority of participants (94%) using ion-exchange techniques. On the other hand the standards used for calibration could contribute to the dispersion of results, especially for histidine and ornithine. The blind samples enabled problems of calibration. of linearity of measurement, of contamination, and of the identification of unusual amino acids to be brought to light.

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Glutamine metabolism in critically ill patients

After trauma patients loose muscle protein and have a negative nitrogen balance. Free amino acids, mainly glutamine, are transported from peripheral tissues to the splanchnic area for oxidation, gluconeogenesis and liver protein synthesis and to rapidly dividing cells such as the immune system.

24 hours after open elective cholecystectomy the intracellular concentrations of free glutamine decreases from 15.0 ± 0.7 to 11.9 ± 0.9 μmol/g ww (mean ± SEM, $p < 0.001$). Still one month after elective surgery the original preoperative level of glutamine is not regained. The glutamine level decreases from 14.4 ± 0.8 in the preoperative state to 9.0 ± 0.6 ($p < 0.001$) on the third postoperative day and to 10.8 ± 0.7 μmol/g ww ($p < 0.05$) thirty days after surgery.

It has been suggested that the protein catabolism observed after elective surgery and trauma is induced by stress hormones and cytokines. In healthy volunteers the concentration of intracellular glutamine shows a similar decrease at 24 hours after a stress hormone infusion from 13.2 ± 0.6 to 9.2 ± 0.4 μmol/g ww ($p < 0.01$), as been described in the postoperative period.

During the first three days after open cholecystectomy the drop in intracellular muscle concentration of free glutamine is not counteracted by conventional total parenteral nutrition (TPN). However, if the TPN is supplemented with either glutamine, the dipeptide alanyl-glutamine or α-ketoglutarate (the corresponding carbon skeleton to glutamine) the loss of muscle glutamine diminishes.

In critically ill patients the intracellular concentration of glutamine in muscle is markedly decreased and is therefore considered as an essential amino acid in this context. Muscle glutamine levels drop early in the course of the critical illness (3.5 ± 0.3 μmol/g ww) and no obvious change is observed after 4–7 days (3.2 ± 0.4 μmol/g ww). The concentration of muscle glutamine in critically ill patients is significantly increased by a isonitrogenous (0.2g N/day) and isocaloric TPN regimen supplemented with α-ketoglutarate (0.28g/kg/day) compared to a control group receiving only conventional TPN.

	Before TPN	After TPN
control group (n = 13)	3.0 ± 0.5	3.0 ± 0.4
αKG-group (n = 6)	3.0 ± 0.3	5.2 ± 1.1 ($p < 0.05$)

In conclusion, TPN regimen supplemented with α-ketoglutarate counteracts the marked reduction in muscle free glutamine concentration in critically ill patients. However, it is yet unclear if the improved muscle glutamine concentration has any benefial effects on the clinical outcome of the critically ill patients.

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The use of the stable isotope ^{15}N leucine for the measurement of the excretion of endogenous protein

The difference between ingested and excreted nutrients in faeces or ileal chyme is referred to as the apparent digestibility. The excreted protein in faeces or ileal chyme can be divided into undigested feed protein and excreted endogenous protein. When only the excretion of undigested feed protein is taken into account the true digestibility can be calculated. The true protein digestibility is a measure of the breakdown of feed protein in the intestinal tract. In order to measure the true protein digestibility, the apparent protein has to be corrected for the amount of excreted endogenous protein. At present the ^{15}N-dilution technique with infusion of ^{15}N Leucine is considered to provide the most realistic figures, although some aspects in this technique need further validation.

With this technique the animals' body protein, including endogenous protein is labeled with ^{15}N. With the aid of the labeled endogenous protein in faeces or ileal chyme a differentiation can be made between excreted nondigested feed protein and excreted endogenous protein. Results obtained show that the true protein digestibility of most feedstuffs is higher than 90%. The lower apparent digestibility is due to the excretion of endogenous protein. The excretion of endogenous protein varies considerably depending which feedstuffs are fed. With feedstuffs like wheat, barley, peas, beans and soya bean meal the excretion of endogenous protein is distinctly higher compared to skim milk powder. The variation in excretion of endogenous protein was found to be dependent upon various components of the feed such as fibres (NDF), trypsin inhibitors, antigenic proteins and tannins. The high true digestibilities indicate that to increase the protein utilisation, it is important to reduce or inactivate those factors that cause increased excretion of endogenous protein. For example, the use of proteases for feedstuffs with a high true protein digestibility is not relevant, because these proteins are already nearly completely digested in the intestinal tract. The results obtained have implications both in animal and human nutrition.

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Amino acid pattern in vitreous humour of human and bovine eye

In starting a study on amino acid and amino derivative content in human subretinal fluid to determine what contribution was made to this by vitreous humour flowing from the wounded retina into the subretinal space we detected their content in human vitreous humour.

Because of the difficulty involved in collecting several samples of human vitreous humour at the same time we performed amino acid analysis of vitreous humour of bovine eye.

Vitreous humour samples were drawn with a syringe from human and bovine eyes removed within six hours of death and kept at 5°C.

Amino acids and amino derivatives of these fluids were detected by using the Beckman System 6300 High Performance Amino Acid Analyzer with the procedure reported by Gueli et al. ([Med Sci Res] 1989)

The amino acid chromatogram of human vitreous humour showed that the highest peak was that of glutamine (range 300–450 nmoles/ml). High levels of taurine, threonine, glycine, leucine, phenylalanine, lysine, valine and alanine were detected in the concentration range of 50–200 nmoles/ml.

Less represented (range 0–50 nmoles/ml) were all the other amino acids and the amino derivatives shown in the chromatogram (Fig. 1A).

The amino acid pattern of bovine vitreous humour (Fig. 1B) was qualitatively and quantitatively similar to that of human eye, except for the higher levels of glutamine, alanine, valine, leucine and lysine detected in human vitreous humour with respect to that of bovine vitreous humour.

We think that the results of this amino acid analysis can be of general interest for knowledge of the composition of the fluids of the eye.

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Relationships of plasma alpha-aminoisobutyric acid to other amino acid and substrate levels and to metabolic patterns during high and low branched chain AA support in sepsis

Abnormally high plasma levels of alpha-aminoisobutyric acid (AIB) is a common feature of sepsis. The relationships between plasma AIB, other plasma amino acid (AA) and indicators of adequacy of metabolic conditions, and the effects of high-dose branched chain amino acid (BCAA) support were explored in two groups of septic patients randomly receiving total parenteral nutrition with 16%-BCAA or 49%-BCAA amino acid solutions (LBC or HBC, respectively; BCAA dose = 0.25 ± 0.10 vs 0.72 ± 0.15 g/Kg/day, $p < 0.01$).

AIB was significantly higher in the LBC group than in the HBC group (23.7 ± 25.6 vs 8.2 ± 5.0, $p < 0.01$). In the LBC group, AIB was directly correlated to plasma levels of glutamine, serine and proline (AA transported intracellularly by transport system A), which explained together 94% of the AIB variability ($r^2 = 0.94$, $p < 0.001$). In the HBC group, most AA levels, and especially "transport system A" AA levels, decreased significantly. In all measurements, AIB was inversely correlated to

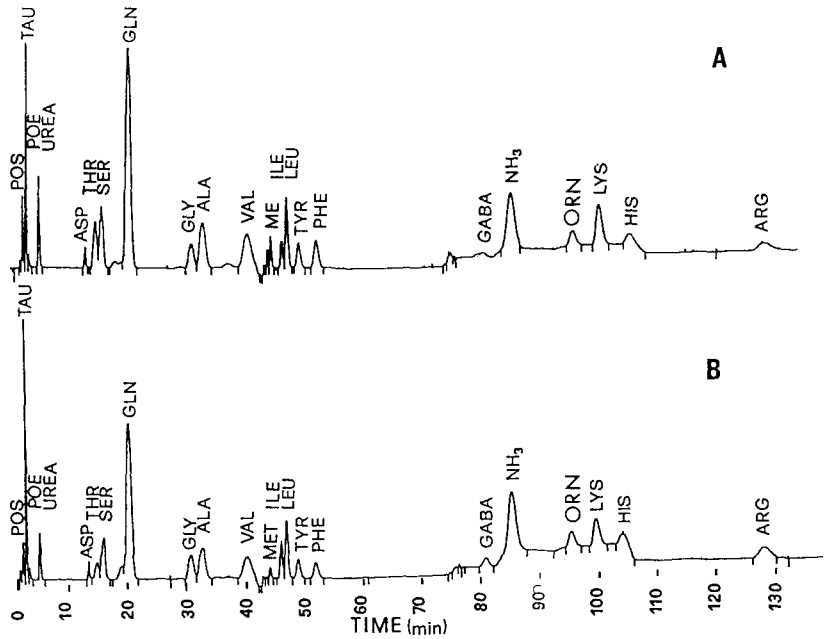

Fig. 1. Amino acid and amino derivative profiles of human and bovine vitreous humour. The amino acids are as follows: POS, phosphatylserine; TAU, taurine; POE, phosphatylethanolamine; ASP, aspartic acid; THR, threonine; SER, series; GLN, glutamine; GLY, glycine; ALA, alanine; VAL, valine; MET, methionine; ILE, isoleucine; LEU, leucine; TYR, tyrosine; PHE, phenylalanine; GABA, γ-aminobutyric acid; LYS, lysine; HIS, histidine; ARG, arginine.

BCAA doses ($r^2 = 0.30$, $p < 0.001$) and clearances ($r^2 = 0.30$, $p < 0.001$) and was irrelevantly affected by other AA doses and clearances. AIB was directly related to plasma levels of lactate ($r^2 = 0.40$, $p < 0.001$) and inversely related to plasma levels of cholesterol ($r^2 = 0.46$, $p < 0.001$). Inverse relationships were also found between AIB and plasma fibrinogen ($r^2 = 0.29$, $p < 0.001$) and between AIB and platelet concentration ($r^2 = 0.38$, $p < 0.001$).

In sepsis, during parenteral nutritional support with high-BCAA dose, abnormal plasma levels of AIB and of other AA transported intracellularly by transport system A tend to normalize. Such an effect suggests a reversal of the peripheral impairment in AA transport, is consistent with an improvement of synthetic processes and is paralleled by the reversal of signs of metabolic impairment.

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Differentiation of the impact of individual amino acids on metabolic variables in critically ill patients

Assessment of metabolic and cardiorespiratory patterns involves an assessment of nutritional-metabolic interactions and of the effect of individual substrates on physiologic variables. An aspect which is generally oversimplified is the different impact of individual amino acids (AA) on metabolic variables. Although the mean nitrogen (N) concentration of AA is thought to be about 1 g.N/6.25 g.AA, the N concentration of individual AA varies considerably. The calculation of nitrogen balance may be affected by this approximation during the administration of modified AA formulae. Although the mean respiratory quotient (RQ) of AA oxidation is known to be about 0.82–0.83, the RQ of individual AA may vary from slightly more than 0.7 to more than 0.9. The combined effect of these approximations in RQ and in N may affect the calculation of nonprotein-RQ from calorimetric measurements, and of fat and glucose balance estimations. Although the mean caloric equivalent of 1 g. AA is known to be about 4 Kcal/g, this figure may vary considerably for individual AA. The gluconeogenic and ketogenic potential, and other relevant characteristics of modified AA formulae are quantifiable on the basis of the sum of the relative contributions of individual AA.

Use of approximative mean figures may be a cause of relevant inaccuracies in determining metabolic variables in critically ill patients in extreme conditions; on the basis of these considerations, precise quantitative data have been used to build up a computer program for an easy determination of exact figures, and for a quantification of errors deriving from the use of approximations.

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Exchange rates of amino acids and metabolism of leucine in malignant colon tumors: results of in-vivo studies using 13C leucine

Introduction: Own studies in man have shown that colon tumors absorbed significantly higher volumes of branched-chain amino acids than healthy colon tissues. For the first quantitative analysis of the metabolism of these amino acids in tumors and especially its extent, we modified and used a 13C-leucine model.

Methods: 15 colon carcinomas were tested during curative resections. Arterlovenous substrate differences in the amino acids were obtained by preparing tumor draining vessels in the mesocolon. The vein was cannulated after ligation and clamping of the lateral vessels. Tumoral perfusion was directly measured by a "venous outflow technique". Protein kinetics in the tumor tissue were analysed by an i.v. 13C-LEU infusion, which was started 90 min. prior to the beginning of surgery. Intraoperative arterial and central venous blood withdrawals and withdrawals from the tumor vein and a deep arm vein allowed for the calculation of protein kinetics for leucine as to the tumors, the peripheral tissues at the arm and the wholebody via the assessment of enrichments and concentrations of 13C-LEU and 13C-KIC and 13CO2.

Results (Mean ± SEM nmol/100 g × min): The weight of the tumor was 80.2 ± 8.7 g. The calculated data on protein kinetics are given in Table 1. A separation of the tested carcinomas into 2 different pathohistological groups (group I: $n = 8$, $G_1 - G_2$, not-mucinous, Group II: $n = 7$, $G_1 - G_2$, 5 mucinous) showed significant differences regarding amino acid exchange and degree of tumoral anabolism (Table 2).

Conclusion: For the first time we could assess quantitative values of tumoral synthesis, the breakdown and the tum, net-balance. The results show that colon tumors absorb large volumes of amino acids and incorporate large volumes in the tumor protein, depending on pathohistology.

Table 1. Proteinkinetics ($p < 0.001$ Tum, vs. P. + WB., WILCOXON-test)

	Tumor	Periphery (P)	Whole-body (WB)
Breakdown	1801 ± 516	177 ± 56	162 ± 10
Synthesis	2170 ± 498	154 ± 65	137 ± 10
Oxidation	162 ± 22	16 ± 2	15 ± 2
Net balance	368 ± 129	−23 ± 22	−15 ± 2

Table 2. Amino acid exchange and net balance diff. in pathohistol. groups (nmol/100 g × min). U-Test acc. to Mann/Whitney

	Total	Group I	Group II	P
All amino acids	374 ± 1999	−3933 ± 2489	5378 ± 1671	0.012
Essential AA	1681 ± 586	111 ± 579	3250 ± 575	0.005
Non essential AA	−1088 ± 1321	−3700 ± 1735	1917 ± 1159	0.012
Branched-chain AA	1523 ± 233	826 ± 131	2205 ± 320	0.003
Leucine	882 ± 127	358 ± 51	1009 ± 198	0.013
Glutamine	1232 ± 259	724 ± 143	1812 ± 452	0.018
Net balance (anabolism)	369 ± 128	122 ± 163	1442 ± 374	0.007

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S-Adenosylmethionine synthesis and its regulation

Rat liver S-adenosylmethionine synthetase (AdoMet synthetase) appears as high-Mr (tetramer) and low-Mr (dimer) forms. Both are inhibited in the presence of GSSG at pH 8. The K_i values are 2.14 and 4.03 mM for the high- and low-Mr forms, respectively. GSH modulates the inhibitory effect of GSSG, but has no effect when added alone. At a total glutathione concentration of 10 mM a K_{ox} of 1.74 was calculated for the high-Mr form, whereas this constant was 2.85 for the low-Mr AdoMet synthetase. No incorporation of ^{35}S-GSSG was observed in either of the enzyme forms, and inhibition of the activity was correlated with dissociation of both AdoMet synthetases to a monomer. The data obtained by GSSG incubation seem to suggest that oxidation leads to the formation of an intrasubunit disulfide.

The AdoMet synthetase sequence presents several consensus motifs for the action of protein kinases. We have studied the possible regulation by protein kinase C of rat liver AdoMet synthetase. Both enzyme forms, tetramer and dimer, are phosphorylated in vitro by this kinase in the same residue, Thr 342 of the sequence. Phosphorylation of the dimer leads to its dissociation, being this the first time that a fully active monomer has been obtained. The kinetics of the monomer have been studied, and a Km_{Met} of 931.9 uM, a Km_{ATP} of 708 uM and a V_{max} of 66.8 nmol/min/mg have been calculated. Alkaline phosphatase treatment of both enzyme forms produces a reduction in their activity with no effect in the oligomeric state. On the other hand, Protein kinase C phosphorylation of the alkaline phosphatase-treated AdoMet synthetase forms leads to the dissociation of the dimer to originate a monomer. Rephosphorylation occurs again in the same residue, Thr 342 of the sequence. This amino acid is the most exposed residue of the whole sequence as deduced from an hydrophaticity profile.

In summary, all the data available in vitro seem to suggest the implication of the ratios GSH/GSSG and protein phosphorylation in the regulation of rat liver AdoMet synthetase.

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Amino acid reactivity and the genetic code

There is much evidence of physico-chemical correspondence between definite codons and coded amino acids. The aminolysis of ester bond is crucial step in the protein biosynthesis in the living cells and many authors suppose, that this reaction have played important role in prebiotic polypeptide synthesis. Therefore idea, that there is correlation between reactivity of amino acids derivatives and structure of respective codons seems to be worth of attention. To check such a possibility we were investigated the model reaction of succinimidyl esters of 18 N-protected proteinaceous amino acids with 4'-anisidine. The free enthalpies of activation ($\Delta G^{\ddagger}$'s) for this reaction were determined. It was shown, that $\Delta G^{\ddagger}$'s of particular amino acids demonstrate periodical dependence on a genetic code arranged in the closed ring by the sequence of one step mutations. The mutations appear as regular series of 2 3 3 3 1 3 3 3 1 3 3 3 1 3 3 3 2 3 3 3 type (the numbers denote a codon position a change took a place). There appear three such "one step mutation periods" in the genetic code. The codons of Gly located between the ends of third and first periods close the one-step mutation ring of the genetic code.

For reaction of acid catalyzed hydrolysis of methyl esters of N-acylamino acids no such a regularity appears what indicates that not the steric factors only influence the amino acid reactivities – genetic code dependence.

Basic Chemistry

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Structure property relationships of amino acids and some dipeptides

A molecular connectivity model of the crystal densities and specific rotations of some natural amino acids and of the longitudinal relaxation rates of some natural amino acids and cyclic dipeptides is presented. The structure-property relationships derived by the aid of this model are very satisfying for the crystal densities and the relaxation rates, while for specific rotations are rather poor. While crystal densities and relaxation rates are better described by a set of three valence molecular connectivity indices $\{D^v, {}^0X^v, {}^1X^v\}$, specific rotations are better described by a set of two simple molecular connectivity indices $\{{}^1X, {}^0X\}$. Use of orthogonal indices, derived from the corresponding ordinary indices shows, in the case of specific rotations and when the orthogonal indices are derived from the $\{{}^1X, {}^0X\}$ set, the possibility to condense the information by the aid of a single high quality descriptor, $^2\Omega$.

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Synthetic formyl-methionyl chemoattractants: A conformation – activity study

Chemotaxis is one of the major processes bringing polymorphonuclear leukocites from the blood to an inflammatory site whether the inflammation is caused by an infectious agent or an allergic stimulus. The discovery that N-formyl peptides of microbial origin are chemoattractants for neutrophils and capable of inducing lysosomal enzyme release as well as superoxide generation, has led to the investigation of the peptide-receptor interaction. The Formyl-Met-Leu-Phe-OH (FMLP) and its methyl ester are the most extensively studied molecules of this group of chemoattractants. On the basis of extensive structure-activity studies, in which the various elements of tripeptides were systematically varied an extended β-sheet structure was originally proposed as the biologically active conformation from spectroscopic analyses of FMLP and active analogs in solution. Recently, the X-ray diffraction structure of FMLP methyl ester indicated the preference of the tripeptide for an open folded

conformation in the crystal state. A folded "active" conformation which allows a strong interaction with the receptor was also proposed on the basis of circular dichroism and i.r. spectroscopy. A γ-bend and a β-bend conformations were suggested as energetically preferable conformations by theoretical studies of FMLP.

In order to obtain more informations on the structural features and conformational preferences of chemotactic N-formyl peptides, we report the synthesis and the results of a biological and conformational study in solution of FMLP methyl ester analogs in which the backbone and/or the aminoacid sequence have been modified. The invastigation was performed to determine the effect induced (a) by the incorporation at position 2 of Formyl-Met-Leu-Phe-OMe of a conformationally restricted chiral residue Xxx [Xxx is: L-Proline, L-Azetidine-2-carboxylic-acid, L-Pipecolic-acid] (b) by substitution of –CO–NH-group with –CO–O-group.

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Photophysical properties of analogues of tyrosine

Time-resolved fluorescence is a very useful tool to study photophysical and conformational properties of biologically active compounds, especially peptides. Fluorescence measurements of proteins and peptides were so far focused mainly on tryptophane. Only little attention was devoted to tyrosine because of measurement difficulties: low absorption, small quantum yield of emission, short time of fluorescence decay and a possibility of proton transfer during excitation. Many native biological peptides, however, contain tyrosine as the only aromatic amino acid. To study the conformation of these peptides and protein spectral properties of tyrosine is essential. We have synthetized analogues of tyrosine and phenylalanine and their derivatives (amides, acetyls, ethers), to study the time of fluorescence decay as a function of chemical structure (influence of amino, carboxyl and hydroxyl groups) and solvent. These experimental data compared with theoretical calculation (molecular orbital or molecular mechanics) should allow to understand better a nature of fluorescence decay of these amino acids and later to accomplish more comprehensive study of peptides containing different chromophores.

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Modelling amino acid metabolism: I General aspects of inherent correlations between parameters in linear and non-linear equations where variables are subject to uncertainty

The starting point for this study was the unexpected finding of a linear correlation between Km and Vmax for *in vivo* hydroxylation of phenylalanine in healthy subjects and in carriers for phenylketonuria (Seakins et al., J Inher Metab Dis 1992; 15: 431–434; Wang et al., J Theor Biol 1992; 155: 485–495). Simulation studies revealed that the correlation was caused by experimental uncertainty in the measurement of the plasma concentration of phenylalanine and the estimate of the elimination rate of phenylalanine. We have noted in the literature that similar correlations have been observed for the elimination of a drug obeying Michaelis-Menten kinetics (Metzler & Tong, J Pharm Sci 1981; 70: 733–737] and parameters from equations used in characterising economic systems. Economists have called such correlations "inherent correlations".

These findings raised the question whether inherent correlations are a common feature of parameters in any mathematical function in which variables are subject to random (experimental) error. As it is unlikely that the procedure used to demonstrate inherent correlations in the Michaelis-Menten equation can be generalised, it was decided to perform Monte Carlo simulation studies on a representative group of mathematical functions relevant to biochemistry.

The approach was as follows: A set of x-values or reference values were drawn from an "ideal" or reference curve (for example plasma concentration against time) which was mathematically defined, for example a bi-exponential decay curve. Random error was added to each of the reference values corresponding to a fixed coefficient of variation (CV) in the range of 1% to 10%. This generated a new set of x-values ("experimental values"). The mathematical function describing the reference curve was fitted to the "experimental" points by a least squares method generating values for the parameters of the equation. This process was repeated a thousand times, to give a set of parameter values and derived parameters for a given CV. The presence of correlations between themselves and the relationships derived from them were analysed by regression analysis.

We found that inherent correlations (i) were a common feature between parameters in non-linear equations where the variable(s) are subject to experimental error, (ii) do not appear to be predictable from the type of equation involved or the mathematical relationship between the (derived) parameters (see also Hjelm & Seakins and Seakins & Hjelm, Abstr 3rd Int Cong Amino Acids, Vienna 1993).

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Modelling amino acid metabolism: II The effect of experimental errors on secondary parameters derived from bi-exponential decay curves

In 1992 we reported (Wang et al., J Theor Biol 1992; 155: 485–495; Seakins, et al., Wang, J Inher Metab Dis 1992; 15: 431–434) the finding of a high correlation between the *in vivo* values of Km and Vmax for phenylalanine hydroxylase in reference subjects and in carriers for PKU and its origin in experimental errors. This prompted us to re-examine the data of Rödjer et al (MD Thesis, University of Gothenburg Sweden 1977) on intravenously administered phenylalanine given as a bolus.

The bi-exponential decay of P-Phe, $C = A^*\exp(-a^*t) + B^*\exp(-b^*t)$, was consistent with a two-compartmental model with phenylalanine being eliminated only from the central compartment (Vc) which corresponded to highly perfused tissues which rapidly equilibrate with the blood plasma. The second, peripheral compartment (Vp), comprised of tissues which are less well perfused. From the values of A, a, B, b and the dosage, the first order transfer (k12, k21) and elimination constants (kel), and the volumes, Vp and Vc were calculated. Using a spreadsheet these constants were examined for the presence of correlations. For reference subjects ($n = 13$) there were 13 statistically significant correlation ($p < 0.05$) and for carriers ($n = 8$) 8 correlations out of a total of 36 correlations.

Simulation studies were therefore performed with experimental error (CV = 1 to 10%) added to the plasma concentration, with a 1000 simulations for each value of CV. The primary

values (A, a, B, b, group 1) were calculated and also the derived values (k12, k21, kel,group, 2; and Vp, Vc, group 3). Correlations for the pairs in groups 1, 2 and 3 were calculated. Out of totally 10 correlations, there were 6 with r-values greater than 0.6.

These results indicate that many of the correlations between primary and derived parameters are linked to uncertainty in the measured variables and do not represent biological correlations.

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Modelling amino acid metabolism: III Inherent correlations between secondary parameters in multi compartmental models

In multi compartmental models of amino acid metabolism a large number of parameters are required to describe the flux of amino acids and their metabolites between compartments. Such parameters are also used to calculate distribution volumes, transfer constants, elimination and turnover rates. For complex models more than twenty parameters can be involved (see Cobelli et al, Am J Physiol 1991; 261: E539–E550).

The experimental data on which these calculations are made usually consists of serial measurements of the plasma concentration of a particular amino acid and sometimes also a metabolite of the amino acid. These measures are not error free and thus it could be expected that both biologically valid correlations and inherent correlations, which are caused by experimental uncertainty, could be present between parameters and distort the interpretation of result.

We have investigated these possibilities by examining data on six volunteers from one study on leucine metabolism for the elimination of the labelled amino acid and its keto-analogue after a bolus injection [Cobelli et al, see above]. Their model consisted of eleven compartments and involved eighteen transfer constants, seven compartmental masses and four leucine flux parameters. The parameters from the fitted decay curves were used to calculate the derived parameters. Correlations, calculated for the 378 possible pairs, were reviewed. In all there were thirty two (8.5%) significant correlations distributed according to P-values as follows:-

P	Number
0.0001–0.0010	2
0.0010–0.0100	10
0.0100–0.0500	20

In addition there were sixteen correlations with a P-value between 0.05 and 0.10. As the statistical analysis was based on data from six individual experiments only, it could be expected that some of these correlations may have reached a higher level of significance had more data been available. r-values linked to significant correlations ($P < 0.05$) varied between 0.810 and 0.987.

Closer examination revealed that transfer rate parameters for reversible and irreversible steps at considerable distance within the model were correlated and that transfer constants were correlated with outcome parameters in an unpredictable fashion.

In the context of biomedical modelling, two types of correlations between parameters may exist, (i) true, intrinsic, correlations in the sense that they represent physiological and biochemical relationships, (ii) correlations due to inherent relationships between variables in the equations made "visible" in situations where experimental uncertainty is linked to them. Both types of correlations could, as indicated by the results above, be present in multi-compartmental modelling.

Interpretation of results would depend on the type of correlation. It is unlikely that a stringent mathematical theory can be devised for classification of the correlations. Methods based on simulation studies would, however, provide a means of identifying inherent correlations (see also Hjelm & Seakins and Seakins & Hjelm, Abstr 3rd Int Cong Amino Acids, Vienna 1993).

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Plasma amino acid correlations. A reinvestigation of the data of Snyderman et al. (1968) on the effects of different protein and amino acid intakes in babies

We have used a spreadsheet (Microsoft Excel 4) to re-examine the data of Snyderman et al (Pediat Res 1968; 2: 131–144). Four groups of infants on isocaloric diets were studied, of which three received varying amounts of protein. These were (in g/kg/day) 1.1, PL (n = 14); 3.0–3.5, PN (n = 29); and 9 g, PH (n = 19). The fourth group received an amino acid mixture equivalent to 3.0–3.5 g protein/kg/day. This group was subdivided into two groups, less than 5 weeks of age, AA1 (n = 35) and older than 5 weeks, AA2 (n = 39). For technical reasons the following amino acids were not measured or are excluded from our statistical analysis for other reasons, Asp, CyS, Gln, Glu, Try and Met (transient hypermethioninaemia), leaving 18 amino acids for investigation. In this abstract we report the results of our studies on correlations between the amino acids.

There are a total of 153 pairs of amino acids and because some of the distributions may not be Gaussian, only those pairs having correlations with $r >= 0.6$, or $P \sim < 0.01$ are considered. Selected pairs were further analysed statistically and graphically.

In the first three groups which received protein, the number of correlations were 20 (PL), 10 (PN), and 50 (PH). The distributions of these correlations varied considerably between these groups, but the correlations between the branched chain amino acids were high throughout and the correlation coefficients (r) were greater than 0.83. The regression coefficients and intercepts for the regression lines for the corresponding three pairs in each group, Leu versus Val, Ileu versus Val, and Ileu versus Leu, were not significantly different from each other, and were therefore combined. With the exception of Gly – Butyrine at 3.5 g protein, all significant correlations are positive.

In the two groups, AA1 and AA2, there were somewhat fewer correlations, respectively 5 and 15 than in infants on protein, and they were smaller in value. Graphical examination of the distributions for the branched chain amino acids showed the presence of outliers which were responsible for the high values of r. The distributions of the correlations were different in the two groups although the concentrations of the plasma amino acids differed little from each other in these two groups.

The finding of these correlations indicates that the systems controlling amino acid metabolism are more complex than previously thought, and that these systems respond differently to the type an amount of diet given. These differences may be pathologically significance and indicate alternative supplementary approaches to monitoring patients on synthetic diets containing free amino acid mixtures.

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Plasma amino acid correlations after intravenous alanine

There is evidence that the concentrations of many plasma amino acids are related under certain circumstances both in healthy subjects [Seakins & Hjelm, Abstr 3rd Int Congr Amino Acids, Vienna, 1993] and in patients with metabolic disorders (Byrd et al., Eur J Paediatr 1989; 148: 543–547).

In this study we have investigated the presence of correlations between the plasma concentrations of amino acids after intravenous loads of alanine (0.25 g/Kg body weight) in healthy adult subjects ($n = 13$), where the plasma concentration of alanine increased to greater than 20 times its physiological concentration before gradually returning to normal (Kay et al., Clin Sci 1987; 72: 187–193). Within-subject correlations between individual amino acids were calculated using a spreadsheet. A two dimensional graphical presentation of the results has been developed for displaying the results on an individual or on a group of subjects.

We observed many significant correlations between individual amino acids after an alanine load, varying from fairly weak ($r < 0.5$) to unexpectedly strong ($r > 0.8$). Two concepts were introduced to characterise the correlations, (i) a *Link ratio*, which is the number of statistically significant correlations between a particular amino acid and other amino acids in a particular individual. (An average link ratio can be calculated from individual ratios), and (ii) a *Consistency ratio*, which is the frequency of statistically significant correlations between specific pairs of amino acids in a group of individuals. The average Link ratio (between 18 amino acids) varied between 0.28 (for Try) to 0.56 (for Asp). Consistency ratios varied from zero (for Lys *vs* Asp) to 1.0 (for Ala *vs* Asp, Try *vs* Asp and Thr *vs* Ser). These results indicate that e.g. Asp is usually correlated with many other amino in a particular individual and that e.g. Thr is always correlated with Ser, a totally unexpected finding, after intravenous alanine. Negative correlations accounted for about 15 per cent of all significant correlations ($p < 0.05$).

The outcome supports the proposal that amino acids are components of a system where concentrations of the amino acids both intracellularly and extracellularly are interrelated to a much larger extent than previously assumed. Animal studies would be required to verify the hypothesis by sampling of amino acids both in plasma and tissues and for elucidating physiological aspects of such a system.

Arginine

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Nitric oxide synthesis from L-arginine by cytokine-stimulated macrophages

Nitric oxide produced in high amounts by the inducible form of NO synthase plays an ambivalent part in the immune system: NO behaves as an effector molecule of macrophage and hepatocyte cytotoxicity against intracellular pathogens, as a mediator of immunosuppression, or as a pathogenic factor in autoimmunity. Biosynthesis and dissemination of such a reactive molecule must require stringent control. It is known from *in vitro* and *in vivo* experiments that NO biosynthesis is under the influence of the cytokine network. IFN-γ, IL-1, TNF are positive regulators of the inducible NO synthase whereas TGF-β, IL-4 or IL-10 were reported to down-regulate NO synthesis. Given the complexity of cytokine interactions, it is difficult to state the real contribution of each cytokine. However, by using specific antibodies, the role of endogenous TNF in NO synthesis induction was demonstrated in murine macrophages stimulated by IFN-γ, or invaded by microorganisms.

At a biochemical level, it was shown that activation of murine macrophages by IFN-γ results in NO-dependent impairment of mitochondrial functions. Furthermore, inactivation of several iron-sulfur enzymes involved in ATP synthesis correlated with the presence of nitrosyl-iron complexes detectable by Electron Paramagnetic Resonance. The activity of two iron-sulfur enzymes was followed after macrophage stimulation: mitochondrial aconitase which catalyses citrate: isocitrate conversion in mitochondria and cytoplasmic aconitase which is also an RNA-binding protein (iron regulatory factor) which regulates synthesis of ferritin and transferrin receptor post-trancriptionally. Activity of both mitochondrial and cytoplasmic aconitase was modulated by NO in response to IFN-γ.

It is likely that the capacity of NO to bind transition metals or thiol groups, located at or close to active site of enzymes or in nuleic acid binding domain of *trans*-regulators, is one of the biochemical explainations for the effects of cytokines on cell metabolism.

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N^G,N^G-dimethylarginine dimethylaminohydrolase in the nitric oxide-generating tissue and cell systems of rats

We previously characterized two enzymes involved in the metabolism of guanidino-*N*-methylated L-arginines in rats. One of them, N^G,N^G-dimethylarginine dimethylaminohydrolase (EC 3.5.3.18) is an unique enzyme, which hydrolyzes specifically N^G,N^G-dimethylarginine and N^G-monomethylarginine to form L-citrulline and dimethylamine, and L-citrulline and monomethylamine, respectively. Both the methylated arginines are known to act as competitive inhibitors of L-arginine: nitric oxide (NO) generating pathway.

In order to elucidate the biological role of the enzyme, we prepared monoclonal antibodies (mAbs) against the enzyme from rat kidney and examined the distribution of the enzyme in rats, in the present study. Four mAbs have been obtained by the fusion of the spleen cells from BALB/c mouse immunized with the sodium dodecyl sulfate-denatured or native enzyme and P3X63Ag8U1 myeloma cells. All the mAbs were shown to bind to the denatured enzyme, but none of them could recognize the native enzyme. The occurrence of the enzyme protein in various rat tissues and cell systems such as peritoneal neutrophils and macrophages was examined using an immunoblotting technique with one of the mAbs. The immunoblotting analyses showed that the enzyme protein are widely distributed in rat tissues, including NO-generating systems such as brain, aorta, peritoneal neutrophils, and macrophages. Since N^G-monomethylarginine and N^G,N^G-dimethylarginine have been suggested to be specific

blockers of the systems generating nitric oxide, the above findings are of great interest in connection with the regulation of the NO production in rats.

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Is the NO-synthase stimulated by NMDA receptors into NTS of the rat?

The baro and Breur-Hering reflexes may be modulated into NTS by several neurotransmitters, including L-glutamate, catecholamines and several neuropeptides. In particular L-glutamate plays an important role in the NTS by activating the NMDA subtype receptors. It has been showed that the activation of these receptors stimulate *in vitro* the NO-synthase. In this paper we thus focused on the dorsal cardiorespiratory neurons in the NTS of rats, and we tried to find out which correlation exists between the NMDA receptor activation and the NO-synthase in the rat NTS. Unilateral microinjections of N-methyl-D-aspartic acid (NMDA; 0.4 and 0.8 mM) into the dorsal cardiorespiratory neurons of the NTS of male anaesthetized (ethyl urethane) Sprague-Dawley rats (250–300 g) elicited a dose-dependent and significant ($p < 0.05$) apnea associated with arterial hypotension. The microinjections of sodium nitroprusside (0.5 and 1 M), a nitric oxide (NO) donor, also elicited a dose-dependent and significant apnea with decrease of blood pressure. A pretreatment into the NTS with the NO precursor, L-arginine (0.5 M), and with the NO-synthase inhibitors, N-nitro-L-arginine (16 mM) and N-monomethyl-L-arginine (15 mM), did not change the NMDA-induced vascular and respiratory effects. These preliminary data suggest that NMDA receptors, in the NTS dorsal cardiorespiratory neurons of anaesthetized rats, may induce apnea and hypotension in a L-arginine/NO pathway indipendent manner.

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Metabolisation of N^{ω} hydroxy-L-arginine by some hemeproteins

Nitric Oxide (NO) is a recently discovered messenger molecule with very important functions in the physiology of mammalian cardiovascular, immune and nervous systems. Biosynthesis of NO involves an oxidation of L-Arginine by NADPH and O_2 catalyzed by different kinds of NO Synthases (NOS) and leads to Citrulline and nitrogen oxides. The N^{ω} hydroxy-L-Arginine (NOHA) is an intermediate in this reaction (eq. 1).

(eq. 1) L-arginine $\xrightarrow[NOS]{NADPH, O_2}$ $R-C(=N-OH)-NH_2$ (NOHA)

$\xrightarrow[NOS]{NADPH, O_2}$ $R-C(=O)-NH_2$ (Citrulline) + NO $\xrightarrow{O_2}$ NO_2^-, NO_3^-

We synthesized this new amino-acid and undertook to study its oxidative metabolism, to know whether other enzymes than NOS are able to catalyse the second step of eq 1.

Rat liver microsomes catalyzed the oxidative denitration of NOHA by NADPH and O_2 with formation of citrulline and nitrogen oxides. The formation of NO could be detected under the form of its cytochrome P450 and P420 Fe (II) complexes by UV-Visible and EPR spectroscopy. Classical inhibitors of NOS failed to inhibit the microsomal oxidation of NOHA to citrulline and NO_2^-. On the contrary, classical inhibitors of hepatic cytochrome P450 (P450) strongly inhibited this monooxygenase reaction.

Liver microsomes from rats pretreated with various inducers of P450 isoforms exhibited different abilities to catalyze the oxidation of NOHA and the particular ability of P450s of the 3A subfamilly was observed.

In the same manner, Horseradish Peroxidase (HRP), a model of peroxidase, catalyzed the oxidation of NOHA by H_2O_2 with concomitant formation of citrulline and NO_2^-.

These results provided evidences that hepatic P450, and other hemeproteins, are able to catalyze the second step of the reaction performed by NOS (eq 1). They suggested another way of NO formation, not involving only NOS, with two steps: (i) the formation of NOHA from L-Arg and NOS in some kinds of cells, and (ii) the uptake of NOHA and its hemedependent oxidation to NO in other cells or tissues. The physiological significance of this pathway of NO formation remains to be established.

Boron Neutron Capture Therapy

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[3] Department of Chemistry, Shinshu University Matsumoto, Japan
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$^{10}B_1$-para-boronophenylalanine administration to human melanoma patients for neutron capture therapy: Boron analytical studies and clinical application

Since our first success in clinical application in 1987, we have treated 11 melanoma patients and 1 lentigo maligna patient by neutron capture therapy (NCT) using DL-$^{10}B_1$-para-boronophenylalanine (^{10}BPA) so far (May, 1993). At the same time, we have investigated various methods of ^{10}BPA administration to melanoma patients in order to obtain selectively higher boron accumulation in melanoma than in surrounding skin and blood. In addition to 12 patients treated by NCT, we also administered ^{10}BPA to 12 patients whose melanoma lesions were surgically removed, with their agreement.

In July 1987, we administered ^{10}BPA · HCl to the first patient by 4 cm distant perilesional injections. In this patient, *in situ* boron assay by prompt gamma-ray spectrometry showed approximately 24 ppm ^{10}B in the tumor, 3 ppm ^{10}B in covering skin and 1.1 ppm ^{10}B in blood, resulting in complete regression of the NCT-treated tumor without skin ulceration.

In the second patient, a slurry of ^{10}BPA in water was orally administered, but it was suggested that a slurry of ^{10}BPA is not well absorbed from the gastrointestinal tract.

Since the third patient, ^{10}BPA has consistently been administered to 22 patients utilizing ^{10}BPA · fructose complex solution (^{10}BPA: 3%, D-fructose: 6.6%) with an adjusted pH of 7.4, which was observed to be much less painful when injected than ^{10}BPA · HCl.

In 10 patients treated by NCT using ^{10}BPA · fructose complex, ^{10}B (our usual dose: 170 mg/kg · BW) was administered either i) by a combination of perilesional injection and systemic administration (subcutaneous injection into buttock, oral administration or drip intravenous infusion), ii) by a combination of intravenous infusion and subcutaneous injection into buttock, or iii) by drip infusion only.

In one male patient bearing recurrent multiple amelanotic metastatic lesions on his right thigh, the boron concentrations in melanomas collected about 1 hour and 4.5 hours after drip infusion of ^{10}BPA (170 mg/kg · BW) indicated approximately 20 ppm and 10 ppm, respectively. Furthermore, in the same patient, who developed multiple metastatic lesions again several months later, ^{10}BPA (50 mg/kg · BW) was injected subcutaneously into the left buttock five times at 3-hour intervals. The boron concentrations in the melanomas resected 7 hours after the last injection ranged from 9 to 18 ppm.

In another patient, to whom half the usual dose of ^{10}BPA (85 mg/kg · BW) was orally administered, the boron concentration in melanomas ranged from 5.5 to 7.1 ppm 19 hours later, while that in blood was 1.5 ppm and that in skin ranged from 1.2 to 1.6 ppm.

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Transport and metabolism of boro-phenylalanine in human uveal cell cultures. Relevance to BNCT

Borophenylalanine (BPA) is considered to be the best boron containing compound for BNCT trials in melanoma cells because of its role as precursor for melanine. Few studies however have been reported on the transport mechanisms and the metabolism of BPA in melanoma cells. In the present study human uveal melanoma cells have been cultured from a primary tumor using standard techniques. Several cell lines have been isolated and characterized but the report will present data only from cell line MK-T. Morphological analysis of this cell line show that cells contain promelanosomes and melanosomes poorly melanotic or empty. BPA is a possible analog of both phenylalanine (Phe) and of tyrosine (Tyr); the uptake of both radioactive aminoacids was then followed as a function of time from 1 min to 36 hrs. Both uptakes were shown to be linear with time and to be inhibited by the presence of various concentrations of BPA (0.45–3.5 mM). The kinetics of the transport of [^{3}H]Phe and of [^{125}I] and [^{3}H]Tyr (measured after 10 sec of incubation) showed a Km of the order of 0.1 mM and a competitive inhibition of both aminoacids with BPA. The results hence suggested that BPA might act as an analog of both Phe and Tyr, even if the transport of Tyr showed a higher affinity for BPA than that of Phe.

Following experiments were devised to ascertain the possible metabolism of BPA. Cells were incubated with 0.45 mM BPA for 16 hrs and the concentration of ^{11}B was measured with a plasma torch (Ionisation Coupled Plasma) in various fractions of the cell homogenate. The results show that in all cases ^{11}B is found in the deproteinated supernatant. No ^{11}B was found associated with soluble or membrane bound proteins.

In conclusion the data show that BPA may be transported as an analog of Phe and Tyr but that it is not metabolized by the cell into proteins nor into melanine since the MK-T cell line is amelanotic.

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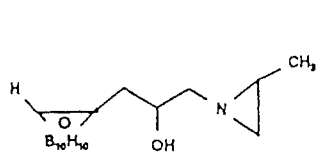
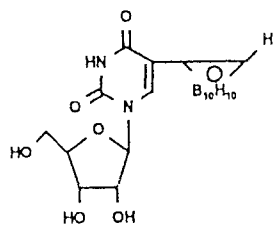
Synthesis and biological properties of new ^{10}B carriers

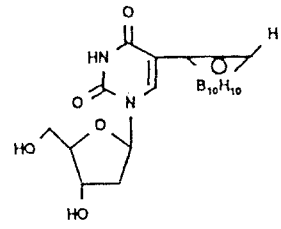
Carborane containing uridine derivatives, $5B_{10}U$, $5B_{10}DU$, $5HB_{10}U$, and $5'Gly5B_{10}U$ have been synthesized via the reaction of decaborane with acetylenic uridines. Methylaziridine derivative bearing carborane (MACB) has been prepared by the reaction of carboranyl epoxide with cuprate of methylaziridine. Water soluble p-boronophenylalanine derivatives, $BPA(OH)_2$ and $BPA(OH)_4$, have been synthesized.



MACB



$5B_{10}U$



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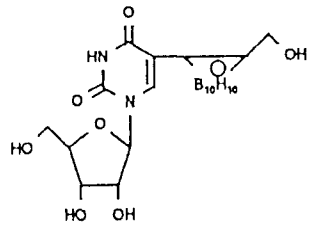

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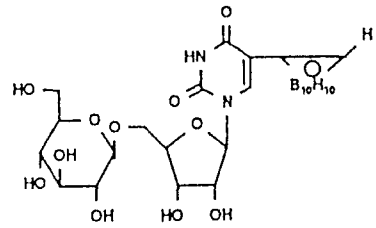
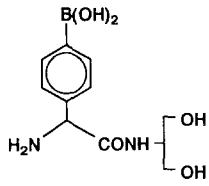
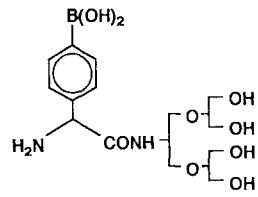


$5'Gly5B_{10}U$



$BPA(OH)_2$



$BPA(OH)_4$

Cytotoxicity of these compounds toward several cancer cells and a normal cell (fibroblast) have been investigated. Uptake of boron atom by cancer and normal cells has been investigated using ICPAES. Biological properties of the newly synthesized carborane compounds will be presented.

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BNCT effect of boro-methylglycylphenylalanine on experimental brain tumor models

BNCT effect is depend on the boron concentration ratio of tumor/normal brain (T/N), and also tumor/blood (T/B). The current boron carrier of boro-captate (BSH) showed the large T/N ratio of ca. 8, however the B/T ratio was around 1 which indicated non-selective uptake into tumor. Also high boron concentration of blood restrict the neutron dose in order to circumvent the normal endothelial damage, especially in the case of deep seated tumor. Phenylalanine analogue of para borono-phenylalanine is a effective boron carrier on BNCT for malignant melanoma. However its concentration in normal brain was reported to be intolerably high for the purpose of BNCT for brain tumors. In order to improve the T/N ratio in brain, boronated dipeptide of boro methylglycylphenylalanine (MGP) was synthesized deriving from boro trimethylglycine. Its killing effect on cultured glioma cell, T98G, and distribution in brain bearing 9L glioma have been investigated. The BNCT effect on cultured cells was nearly triple in comparison with boro-captate. The neutron dose yielding 1% survival ratio were 7×10^{12} nvt for MGP and 2×10^{13} nvt for BSH respectively, after the boron loading incubation for 16 hrs in the same B-10 concentration of 20 ppm. Alpha auto radiography on rat 9L brain tumor revealed that the T/N ratio was almost the same in the case of BSH, 16 hrs after intraperitoneal loading. These data suggests that MGP is the possible candidate for BNCT.

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Boronated amino acids and peptides for neutron capture therapy

We are interested in the synthesis, pharmacological, and neutron capture properties of boron-containing intermediate antimetabolites. These include analogues of amino acids, peptides, boranophosphates, and more recently, the boron analogues of nucleosides and nucleotides.

This presentation will focus on the preparation and properties of select boronated amino acids and peptides and their potential use in BNCT. Emphasis will be given to a number of dipeptides prepared from a boronated amino acid and normal aromatic amino acids containing tyrosine and phenylalanine. Cell survival curves of *in vitro* irradiation of cancer cells previously exposed to the boron compounds will be presented. Biodistribution studies in rodents will be discussed.

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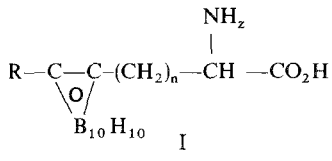
Carboranyl amino acids with potential use in BNCT

Currently, much interest is being paid to the synthesis of boron compounds which could be delivered selectively to tumour cells either *per se* or by use of targeting strategies, including the use of monoclonal antibodies, growth factors and liposomes. α-Amino acids containing the *closo*-1,2-$C_2B_{10}H_{11}$-carborane cage are potential boron compounds for this purpose.

In this contribution styrategies for the asymmetric synthesis of both enantiomers of amino acids of type I are described and exemplified by the synthesis of **Ia–Id**.



	R	n
Ia	H	1
Ib	H	3
Ic	CH_3	3
Id	CH_3	1

The amino acids are obtained either via a) alklyation of chiral glycine enolate equivalents or b) electrophilic amination of a chiral enolate of a bornanesultam derivative of a carboranylalkanoic acid. In the former method the chirality is introduced at one of two different stages in the synthesis depending on the nature of the substituent R and the value of n: a) (i) a haloalkyl carborane or a) (ii) an acetylenic halide is allowed to react with the glycine enolate. In case a) (ii) the reaction product is converted to a carborane by reaction with the bis-acetonitrile complex of decaborane. The chiral glycine equivalents used are the imidazolidinone introduced by Fitzi and Seebach (1988) and the bornanesultam derivative introduced by Oppolzer et al. (1989).

The amino acid carboranylalanine (**Ia**) easily undergoes degradation to a roughly equimolar mixture of the diastereomeric *nido*-analouges when the acid is heated in water.

R. Massarelli[1], R. Belkhou[1], P. Pham[2], N. Jasner[1], J. Sahel[3], and J.-C. Abbe[1]

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[3] Ophtalmology Clinic, CHU, Strasbourg, France

Transport and metabolism of boro-phenylalanine in human uveal cell cultures
Relevance to BNCT

Borophenylalanine (BPA) is considered to be the best boron containing compound for BNCT trials in melanoma cells because of its role as precursor for melanine. Few studies however have been reported on the transport mechanisms and the metabolism of BPA in melanoma cells. In the present study human uveal melanoma cells have been cultured from a primary tumor using standard techniques. Several cell lines have been isolated and characterized but the report will present data only from cell line MK-T. Morphological analysis of this cell line show that cells contain promelanosomes and melanosomes poorly melanotic or empty. BPA is a possible analog of both phenylalanine (Phe) and of tyrosine (Tyr); the uptake of both radioactive aminoacids was then followed as a function of time from 1 min to 36 hrs. Both uptakes were shown to be linear with time and to be inhibited by the presence of various concentrations of BPA (0.45–3.5 mM). The kinetics of the transport of [^{3}H]Phe and of [^{135}I] and [^{3}H]Tyr (measured after 10 sec of incubation) showed a Km of the order of 0.1 mM and a competitive inhibition of both aminoacids with BPA. The results hence suggested that BPA might act as an analog of both Phe and Tyr, even if the transport of Tyr showed a higher affinity for BPA than that of Phe.

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In conclusion the data show that BPA may be transported as an analog of Phe and Tyr but that it is not metabolized by the cell into proteins nor into melanine since the MK-T cell line is amelanotic.

Analysis

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Use of mass spectrometry for the identification of peptides in cheddar cheese

Peptides found as a result of proteolytic activity during cheddar cheese ripening contribute significantly towards the final flavour which develops in the ripened cheese. Relatively few of these peptides have been identified; traditional techniques for the separation and identification of peptides have proven to be tedious and time-consuming. Recent developments in reversed phase high performance liquid chromatography (RP-HPLC), mass spectrometry (MS) and MS-MS provide excellent opportunities for the separation, recovery, identification and confirmation of the presence of these peptides as well as their origin. This type of information could lead to a more complete understanding of the contribution of various peptides to the flavour of ripened cheese.

In the present work, the peptides fraction of defatted cheddar cheese was solubilized and recovered as the TCA-soluble fraction, this peptide fraction was separated into individual peptides by RP-HPLC. The conditions of separation were as follows: C18 column (0.46 × 25 cm length, VYDAC), 2-buffer gradient elution system; buffer A- 0.1% TFA in water, buffer B- 0.1% TFA 70/30 acetonitrile/water, starting at 10% B and increasing to 70% B at a flow rate of 1 mL/min; UV detection at 210 nm. Separated peptide fractions were analyzed by MS for molecular weight determination using a triple quadropole mass spectrometer (API III, LC/MS/MS system, Sciex, Thornhill, Ontario, Canada) and

for amino acid sequence by MS-MS. The RP-HPLC profile of the peptide extract showed in excess of fifty peaks. However, it was likely that not all were peptides since neither the extraction nor the detection procedures were specific for peptides. Other nitrogenous compounds could also be present in the TCA soluble fraction. In addition, in many instances, a single peak obtained by RP-HPLC showed the presence of more than one molecular component when analyzed by MS. In cases where a single molecular component was found to be represented by a peak on the chromatogram, MS-MS was carried out successfully to determine the amino acid sequence of the peptides. Whenever an amino acid sequence of a peptide was obtained, a "matching" of the peptide was carried out against the various caseins to determine the origin of the peptide. The following peptides and their origin were confirmed: (1) T-V-Q-V-T-S-T-A-V from κ-casein, (2) Q-P-V-L-G from β-casein, (3) F-V-A-P-F-P-Q-V-F-G-K-E from α-casein and (4) F-V-A-P-F-P-Q-V-F from α-casein.

J. Csapó[1], S. Folestad[2], A. Tivesten[2], and Zs. Csapó-Kiss[1]

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The use of mercaptoethanesulfonic acid as a protecting and hydrolysing agent at elevated temperatures for rapid determination of the amino acid composition in protein

The problems of the rapid and accurate determination of the tryptophan content in polypeptides, foods and feed protein has not yet been perfectly solved. During the last twenty years attempts have been made to eliminate the destruction of tryptophan residues and in the latest time some hydrolysis methods were developed at elevated temperature and by means of microwave technology. In the present work we used 3 M mercaptoethanesulfonic acid (MES-OH) at 160–170 and 180°C for the hydrolysis of pure proteins, free tryptophan and milk powder with high milk sugar content. Different temperatures and different hydrolysis times were compared under standard conditions. Our aim was to minimise side reactions during 3 M mercaptoethanesulfonic acid hydrolysis for the best recovery of the single amino acids (especially tryptophan and methionine). The following materials were used for testing the hydrolysis methods: Bovine ribonuclease, lysozyme, citochrom C, free tryptophan and mare's milk powder.

In conclusion, high temperature hydrolysis with 3 M mercaptoethanesulfonic acid was successfully applied for the amino acid analysis of pure proteins and milk powder with high carbohydrate content. In this way a rapid method is obtained which yields a complete hydrolysis of protein and also enables determination of all protein amino acid including tryptophan. In the original method the protein was hydrolysed by 3 M MES-OH at 110°C for 24 and 72 h similar to the conventionally used 6 M HCl hydrolysis. In this report we state that the higher temperature and the shorter time give such results that are very similar to the original method. Moreover, the results for tryptophan and methionine when hydrolysed at 160–170°C for 15–30 min were even better than those obtained with the original method. The great disadvantage of this method when using an ion exchange column chromatography method, that the MES-OH reduces cystine to cysteine, which appears in the chromatogram in the place of proline and this, particularly at proteins with high cystine content, may interfere with the proline determination.

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[3] Department of Marine Geology, University of Göteborg, Göteborg, Sweden

Use of amino acid racemization for fossil age determination

A method based on amino acid racemization was developed and evaluated for determination of the age of fossil bones. One hundred fossil bone samples of known age from Hungary were collected and analysed for the D- and L-amino acid content. Because racemization of amino acids is not only age dependent but is also affected by temperature, pH and metal content of soil, these factors were corrected for by using C-14 age of the bone samples. This age had previously been determined by a corrected C-14 method. By plotting the D/L ratio as a function of time for the samples of known age, and by using the half life of racemization, calibration curves were established which subsequently were used for age estimation of fossil samples of unknown age. For this purpose, the D/L ratios of 2–3 amino acids were determined for each unknown sample and then the mean value of ages, estimated on the basis of the calibration curves, was then considered as the age of the specific fossil sample.

In the application of this method to dating of several Hungarian fossil bone samples, there were usually negligible discrepancy between the age estimated from the amino acid racemization with the age obtained from C-14 dating. Notably, a high degree of caution were considered in the sample selection and sample preparation. Thus, unknown samples were mainly of similar origin as those for which the calibration curves had been made. The sample preparation procedure was also kept the same in detail for the samples of both known and of unknown age. In this way, the method proved to give very reliable results and may therefor be used as a general scheme in other dating applications. However, our calibration curves should not be used then because other samples may have been subjected to different environmental conditions (other temperatures, pH. etc.) than the samples in this study. In those applications, other calibration curves should be derived for each different environment.

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PRTB, Roche Research Gent, Belgium

Stoichiometry determination of two-component protein complexes by amino acid composition analysis

Knowledge of the stoichiometry of two-component ligand/receptor or antigen/antibody complexes contributes to the elucidation of the biological response mechanism and facilitates structure-based studies. In comparison with other known methods of stoichiometry determination, as chemical cross-linking, size exclusion chromatography or ultracentrifugation, amino acid composition analysis is the method of choice, if pure protein components are available.

Recombinant ligands and their corresponding receptors produced in prokaryotic or eukaryotic expression systems were investigated. The stoichiometry or interaction between human and mouse interferon γ/interferon γ receptor, human tumor necrosis factor α and β/tumor necrosis factor receptor type A and

human interleukin 5/interleukin 5 receptor were determined by combining amino acid analysis with a least square fitting method. This technique proved to be very reliable and fast and required only small amounts of protein. The method can also be employed for other two-component complexes, like the determination of the loading of a peptide immunogen on a protein carrier.

Polyamines

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A possible role of poliamines in the modulation of the biological activity of the Eukaryotic Initiation Factor 5A

Hypusine [N^{ε}-(4 amino 2-hydroxybutyl) lysine] is present in all eukaryotic cells so far examined. This unusual basic amino-acid occurs predominantly in one single protein, the Eukariotic Initiation Factor 5A (eIF-5A).

Its formation occurs by a series of post-translational reactions by which the butylamine moiety deriving from spermidine is first transferred to the ε-amino group of a specific lysyl residue of the eIF-5A, thus forming the deoxyhypusine.

This intermediate is not normally accumulated in cells, but immediately hydroxylated at the carbon 2 of the incoming group. This final step in hypusine formation is catalyzed by deoxy-hypusine hydroxylase. This enzyme has been partially purified from rat testis and some of its characteristics have been reported.

The role of hypusine in cells is not completely clarified so far. Neverthless in our recent studies we have evidenced a close relationship between hypusine formation and cellular proliferation. In fact rapidly growing cells, as well as proliferative organs, were found to be rich sources of hypusine. Furthermore we have investigated on the correlation between structural modifications of eIF-5A induced by hypusine and its biological function.

The observation that growing cells syntetized significantly higher levels of hypusine than resting cells suggests that hypusine might play some specific role in cell growth and differentation. Moreover the spermidine-dependent biological pathway by which the amino-acid is formed, and the strict relationship of the hypusine-containing protein (eIF-5A) with protein biosynthesis may represent a key manner by whych the polyamines affect regulation of cell proliferation. Indeed this raises the possibility that such a post-traslational modification modulates the actvity of eIF-5A as an initiation factor, thereby contributing to the regulation of translation.

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The polyamine spermidine modulates the NMDA-induced cardiovascular changes in the PAG area of freely moving rats

The NMDA subtype receptor possesses besides the glutamate recognition site other allosteric sites which may modulate the receptor function. Among these there is the polyamine site that, depending on the concentration, may inversely modulate this glutamergic subtype receptor. Recent reports *in vitro* have shown that polyamines spermine (SP) and spermidine (SPD) at micromolar concentrations positively modulate the activity of the NMDA receptors. On the contrary, higher millimolar concentrations of the same polyamines negatively modulate the NMDA receptor functions. However knowing that SP and SPD have multiple concentration-dependent effects on the NMDA receptors *in vitro*, only little information about the polyamine-NMDA receptors' interaction in the whole animal is currently available. We therefore planned to evaluate whether SPD plays a modulatory function on the NMDA receptors at the level of the pressor neurons within the midbrain PAG matter of freely moving rats.

Male Sprague-Dawley rats, weighing 250–300 g, were used. On the day of the experiment, catheters were placed in a femoral artery and vein of each rat, under 2% halothane anaesthesia. The femoral artery was catheterized for direct measurement of arterial blood pressure, with a pressure gauger transducer, displayed on a Hellige polygraph. The jugular vein was catheterized for the systemic administration of heparine or saline. For the direct intracerebral administrations a stainless steel guide cannula was implanted into the latero-caudal periaqueductal gray (PAG) area under ketamine anaesthesia (100 mg/kg i.p.), two days before experimentation, applying the coordinates of the atlas of Paxinos and Watson (measured from the bregma AP: −7.8; L: 0.5; V: 4.5). The intracerebral microinjections were conducted with a stainless steel fine cannula (o.d. 0.6 mm).

We investigated the effects of polyamine spermidine (SPD) (0.01 to 1 μg/rat) on the hypertension induced by N-methyl-D-aspartate (NMDA) (0.1 μg/rat) microinjected into the PAG area of freely moving rats. The pretreatment with the low dose of SPD (0.01 μg/rat) significantly increased the NMDA-induced hypertension. On the contrary, the highest doses of SPD (0.1 and 1 μg/rat) significantly decreased the NMDA-induced cardiovascular changes. SPD alone did not modify the arterial blood pressure. Arcaine (1 μg/rat), a putative antagonist at the polyamine recognition site on the NMDA receptor, microinjected into the PAG area prevented the negative but not the positive modulatory effects of SPD on the NMDA-induced cardiovascular modifications. The pretreatment with SPD did not change cardiovascular effects induced by quisqualic acid (QUIS), a non-NMDA receptor activator. These data, in agreement with the *in vitro* results, suggest that also *in vivo*, at the level of the PAG area, the polyamines have multiple actions on the NMDA receptor.

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Polyamines in diabetic erythrocytes

Variations of erythrocyte polyamine content have been found in patients with insulin dependent diabetes mellitus (IDDM, type I) with complicances such as nephropathy and retinopathy; according to other authors sera from diabetic patients induce an enhanced *in vitro* proliferation rate of fibroblasts, and hence an increased spermidine/spermine ratio in these cells. Polyamines might thus play some role in diabetes, but data on their behaviour in this pathology are as yet still poor, and are in any case restricted to IDDM. We have therefore started a study

devoted to assay polyamine levels in erythrocytes of patients with IDDM and non-insulin dependent diabetes mellitus (NIDDM, type II), matched by sex and with three different ranges of metabolic control as judged by % HbA_{1C} concentration. The study will include a longitudinal follow-up of IDDM and NIDDM patients at their first diagnosis as diabetic subjects. Transglutaminase (TGase), as the enzyme that can accept polyamines as substrates, will also be determined in erythrocytes, because its activity appears to be influenced by oral antidiabetic drugs.

Our first data indicate no significant variation of TGase activity among groups under study. Levels of putrescine are apparently more elevated in IDDM as compared to NIDDM erythrocytes and finally differences are also encountered in spermine concentration between type I and type II diabetic subjects. Our data do not allow at the present time any correlation between polyamine levels and diabetic complicances.

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Polyamine levels in the cam plant *Opuntia Ficus Indica* (Miller)

This research is related to the possible role of polyamines in an obligatory CAM (Crassulacean Acid Metabolism) plant, *Opuntia Ficus Indica* (Miller), widely spread in harid habitats, in order to understand the osmoregulatory mechanisms adapted by plants in arid environments for survival and growth.

The adaptation of plants to environmental changes implies their capability for metabolic variations. Maintenance of sufficient hydration and turgor, which is under hormonal control, is essential to a normal functioning of the biochemical processes involved in survival and growth. Polyamine levels have been shown to increase in response to various abiotic stress condition in cereal leaves and in a non obligatory CAM plant where CAM activities were induced by exposing plants to long day light conditions.

However, no scientific report on endogenous polyamines and obligatory CAM plants under natural field conditions and their relationship to different physiological parameters has been published. This work will present the first results on this topic. Values of endogenous polyamine levels in *Opuntia* will be related to parameters such as CAM activity, age of cladode and osmotic status.

Our observations on CAM activity in cladodes of different age show a very low nocturnal malic acid synthesis in the cladodes from one week to one month of age; putrescine, the most represented polyamine in this species, evidenced higher values in the first stages of growth. Putrescine content also increased during the onset of the buds on one year old cladodes. Furthermore, during cladode growth, variation of spermidine and spermine content showed a pattern similar to that of putrescine, while cadaverine was present in lesser quantity with a variation pattern opposite to that of the other polyamines.

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Modulation of glutamate receptor function in cerebellar granule cells cultured in vitro

Cerebellar granule cells constitute an excellent model system to analyze conditions that modulate the expression/and or function of glutamate receptors and their involvement in excitotoxicity. We have recently reported that when these neurons are grown in the presence of a serum protein complex named NOAC (neurite outgrowth adhesion complex) they form an intense network of neurites, survive for long periods in vitro, express several phenotypic markers typical of neurons, but exhibit a marked resistance to the otherwise lethal action of excitatory aminoacids (EAAs). We found that when these neurons are grown in the presence of NOAC plus human recombinant IFG-I (25 ng/ml) they express functionally active glutamate receptors and become fully sensitive to the toxic action of glutamate and others EEAs. The action of IGF-I is concentration (half maximal effect at 1.5–3.0 ng/ml), time dependent (half maximal effect 2–3 days of culture) and is rapidly reversible (t1/2 = 60 min.) after removal of IGF-I. The "sensitizing" action of IGF-I is accompanied by the appearance of glutamate-activated CA++ permeable channels. On the basis of these findings and of other experiments to be reported we postulate that the constitutive phenotype of cerebellar granule cells is EAA− (resistant to glutamate and other EAAs) and becomes EAA+ (sensitive to glutamate) in the presence of IGF-I and possibly of other polypeptides present in serum and in other biological fluids. The initial characterization of such polypeptide(s) will be presented and discussed on the ligth of their possible relevance to physiological or pathological conditions.

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Kinetics and calcium specificity of polyamine uptake in carrot protoplasts

Recently, evidence has increased for both long and short distance transport of polyamines in higher plants. In order to eliminate the polyamine interactions with the cell wall, which represent an obstacle to polyamine translocation across the plasmalemma, uptake was studied by utilising protoplasts of carrot taproots.

Polyamine uptake at the cellular level is very rapid, reaching saturation after 2 min. The polyamines taken up by protoplasts are mainly compartmented into the vacuole and the transport process is specifically stimulated by Ca^{2+} up to 1 mM, even though the molecular mechanisms by which this cation exerts its effect on polyamine uptake is still unclear. The Ca^{2+} specificity was confirmed by the fact that La^{3+} could only partly substitute calcium, while Mg^{2+} and K^+ at concentrations up to 10 mM did not affect polyamine uptake.

In order to better understand the role that Ca^{2+} plays on polyamine transport process across the plasmalemma, its influence on the kinetic parameters of polyamine uptake was investigated and the effect of Ca^{2+}-channel blockers was studied.

Moreover, for a better characterization of polyamine transport system into carrot protoplasts, a number of metabolic inhibitors was tested.

Spermidine uptake in dependence on the external concentration was biphasic, both in the absence and in the presence of Ca^{2+}; in the first case, saturation was reached at 0.1 to 0.25 mM (K_m = 43 μM, V_{max} = 1.8 nmol mg $prot^{-1}$ min^{-1}) and a linear system from 0.25 to 50 mM appeared; when 1 mM $CaCl_2$ was present, the V_{max} rose from 1.8 to 23.4 nmol mg $prot^{-1}$. min^{-1} and the K_m value became also higher (188 μM). A similar effect, even though less pronounced, was found with regard to putrescine uptake. This suggest the existence of two transport systems, one of which activated by Ca^{2+}, with different characteristics of affinity and capacity.

Putrescine uptake was an energy-dependent mechanism, thus confirming what previously observed in carrot protoplasts with regard to the other two polyamines. Metabolic inhibitors strongly inhibited the transport process and their effect was much more evident on Ca^{2+}-activated uptake, the plasmalemma ATP-ase and a protein with sulfidrilic groups were probably involved.

With regard to the extent of stimulation exerted by Ca^{2+} on polyamine uptake, it varied from 1 to 7.5 times, although an activation between 2.6 and 5 times was more frequently observed. This was probably due to a different sensitivity of carrot protoplasts towards the cation.

Ca^{2+}-channel blockers, such as La^{3+}, Gd^{3+} and verapamil, preincubated before adding Ca^{2+}, almost completely abolished the stimulatory effect exerted by Ca^{2+} on putrescine uptake; the major inhibitory action of gadolinium and verapamil respect to lantanum was confirmed by their higher specificity as Ca^{2+}-channel blockers. On the contrary La^{3+}, when supplied alone, showed a little activation on putrescine uptake, so mimicking somehow the effect of Ca^{2+}. This suggest that Ca^{2+} could activate the polyamine transport process both by penetrating into the cell and by acting at surface level.

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Control of fungal plant pathogens by inhibiting polyamine biosynthesis

The diamine putrescine and the polyamines spermidine and spermine are ubiquitous in nature and are essential for cell division. However, while polyamine biosynthesis in animal and most fungal cells proceeds via the decarboxylation of ornithine, in plant cells polyamines are also synthesized from arginine. Theoretically therefore, specific inhibition of polyamine biosynthesis in fungal cells should inhibit fungal growth without affecting the host plant. Indeed, research over the past six years has shown that the polyamine biosynthesis inhibitor – difluoromethylornithine (DFMO) can control certain fungal infections with no effect on plant growth. Although much information now exists on the use of substrate analogues like DFMO on polyamine biosynthesis in fungi and effects on fungal infection, no data exist on the use of product analogues as inhibitors of polyamine metabolism.

More recently, DFMO and the putrescine analogue keto-putrescine, were shown in glasshouse experiments to provide substantial control of powdery mildew (*Erysiphe graminis*) and brown rust (*Puccinia hordei*) infections in barley, and rust (*Uromyces viciae-fabae*) and chocolate spot (*Botrytis fabae*) infections in broad bean. In a field trial with spring barley, DFMO used at 375 g/ha controlled mildew early in the season at least as well as a mixture of 120 + 192 g/ha flutriafol + carbendazim. The fungicidal activities of these two compounds appear to be related to a pertubation in polyamine biosynthesis, but although DFMO and keto-putrescine affected polyamine biosynthetic enzymes differently, they both reduced spermidine concentrations in the oat stripe pathogen, *Pyrenophora avenae*.

To date, most studies of inhibitors of polyamine biosynthesis as possible fungicides have concentrated on use of enzyme-activated inhibitors e.g. DFMO. Pertubation of polyamine biosynthesis using polyamine analogues might provide a useful alternative to the development of new fungicides. Since inhibition of polyamine biosynthesis is a novel mode of fungicidal action, such inhibitors could provide a useful addition to the existing crop protection portfolio.

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5′-Methylthioadenosine phosphorylase deficiency in cancer cells: A marker of a new tumor suppressor gene

Malignant transformation is due to either the somatic activation of cancer-promoting genes and the germline or to the functional inactivation of tumor suppressor genes. While the search for dominant oncogenes is greatly facilitated by their ability to transform appropriate host cells, the identification of tumor suppressor genes is remarkably complicated by the lack of strong selection procedures. However, extensive karyological analyses along with detailed molecular genetic studies by linkage techniques and RFLP, have shown that nonrandom loss of specific genetic material occurs in a large number of cancers and is probably involved in the pathogenesis of malignancy.

In this scenario, it is of interest the discovery that a high percent of human cell lines derived from various tumors and of specimen from acute lymphoblastic leukemias and gliomas are devoid of 5′-deoxy-5′-methylthioadenosine phosphorylase (MTAase) activity. Conversely, this enzymatic activity has been found in all normal tissues and cell lines of nonmalignant origin investigated so far.

By means of mouse-human somatic cell hybridization studies, the putative gene for the phosphorylase has been mapped at the 9pter-9ql2 region. Since nonrandom abnormalities at 9p have been reported in several malignancies (including hematopietic cancers, gliomas, non-small cells lunga carcinomas, melanomas), it can be hypothesized that a chromosomal aberration encompassing the phosphorylase gene and the putative tumor suppressor gene might be responsible for the occurrence of the enzymatic defect exclusively in malignant cells. It should be underlined that the presence of a recessive oncogene on the short arm of chromosome 9 has also been suggested by chromosomal microcell transfer studies.

Aim of our study has been to investigate the molecular mechanisms that result into the enzyme deficiency in malignant cell lines and tumor specimens of hematological origin. The results obtained, by employing both the radiochemical assay of the enzymatic activity and the immunochemical estimation of the protein indicate that the absence of the MTAase activity is due to genetic alteration(s) that hamper(s) the phosphorylase expression. These results along with Literature data strongly support the idea that a tumor suppressor gene is localized on the 9p chromosome and that this recessive oncogene maps very near to that codifying for MTAase.

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Structure-activity studies on insect adipokinetic/ hypertrehalosaemic peptides

In recent years, much attention has been focused on neuropeptides synthesised and stored in intrinsic neurosecretory cells of the corpora cardiaca of insects, which are involved in the regulation of lipid and carbohydrate mobilization, mainly during flight (Gäde, 1992a). Such compounds, collectively known as adipokinetic and hypertrehalosaemic neurohormones, have been found in all major orders of insects investigated so far (Gäde, 1990). The peptides may have diverse functions in different insect species, but they are all structurally related and are therefore grouped into a hormone family. Structurally, the peptides have the following common features: they are 8 to 10 amino acids long; the N-terminus is blocked by pyroglutamic acid; the C-terminus is amidated; aromatic residues reside at positions 4 (Phe or Tyr) and 8 (Trp), and Gly is always at position 9 (Gäde, 1991, 1992b). With the exception of two peptides, which contain an Asp residue at position 7, all other members of the AKH/RPCH-family are not charged under physiological conditions. Since nature provided a large number of "bioanalogues" of this family, these were used for structure-activity studies in two bioassay systems. In the migratory locust, lipid mobilization was determined; whereas in the American cockroach, carbohydrate release into the blood was monitored. The blocked termini were apparently important for activity in both systems: the ED_{50} values were at least 100-fold higher when the N-terminal pGlu residue was missing, or when the C-terminus was a free acid. At position 2, an Ile residue was accompanied with a 15-fold (cockroach) or 160-fold (locust) higher ED_{50} and a peptide with Tyr^4 (instead of Phe^4) and Asp^7 (instead of Asn^7) had very little activity and never reached more than 50% of the activity of the native peptides. Another feature was that peptides which had Ala, Ser or Thr at position 6 instead of Pro, had 20- to 50-fold higher ED_{50} in the cockroach and not always reached the maximum response. In the locust, those peptides gave no equivocal results: also, some achieved only little activity, others had low ED_{50} values and reached the same maximum response as the native peptide. It is thought that amino acids 5 to 8 form a β-turn and, therefore, changes at those positions may affect receptor binding much more strongly than at other positions.

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Identification of the motilin pharmacophore through single amino acid substitutions

Motilin is a 22 amino acid polypeptide (FVPIFTYGELQRMQEKERNKGQ) that regulates fasting gastrointestinal motility patterns through stimulation of discrete receptors on gut smooth muscle cells and enteric neurons. Exogenous administration of the hormone accelerates intestinal transit time and enhances gastric emptying in healthy human subjects. In addition, motilin infusion stimulates the emptying of solids and liquids in patients with diabetic gastroparesis.

Previous research demonstrated that the N-terminal tetradecapeptide of motilin retained most of the binding affinity and biological activity of the full molecule. Subsequently, alanine and D-amino acid scans of this bioactive sequence identified Phe^1, Val^2, Ile^4 and Tyr^7 as critical residues for motilin agonist activity. In order to more clearly define the physicochemical basis for the high affinity interaction between motilin and its receptor, we have examined several series of analogs of [Leu^{13}]motilin (1–14) (IC_{50} 4.4 nM) in which structural features of these key amino acid residues have been systematically modified. Compounds were assayed in a motilin receptor binding assay employing a rabbit antral homogenate. Biological activity was measured in a contractility assay using rabbit duodenal smooth muscle strips.

Acetylation of the N-terminal amino group {[N-AcPhe^1, Leu^{13}]motilin (1–14)} or substitution of this functionality by hydrogen {[des-NH_2-Phe^1, Leu^{13}] motilin (1–14)} afforded analogs with significantly reduced in vitro potencies (IC_{50}s 26 and 110 nM, respectively). In contrast, biological activity was relatively insensitive to modifications of the Phe^1 side chain, with the caveat that introduction of hydrophilic functional groups {[β-Pal^1, Leu^{13}]motilin (1–14)} adversely affected binding affinity (IC_{50} 56 nM). Replacement of Val^2 or Ile^4 by a variety of natural and unnatural amino acids produced several highly active peptides with potency primarily a function of the lipophilicity of the side chains (IC_{50}s 2.8–3.5 nM).

Minor structural alterations of Tyr^7 were not tolerated unless the modified side chain contained both an aromatic ring and a hydrogen bond donor. For example, deletion of the aromatic hydroxyl group {[Phe^7, Leu^{13}]motilin (1–14)} or saturation of the aromatic ring {[Cha^7, Leu^{13}]motilin (1–14)} was detrimental to in vitro bioactivity (IC_{50}s 6.3 and 174 nM, respectively), whereas incorporation of the 3-indole ring of tryptophan {[Trp^7, Leu^{13}]motilin (1–14)} produced an analog that was more potent than the parent peptide (IC_{50} 3.0 nM).

Based on these structure-activity relationships, the physicochemical features responsible for the expression of motilin agonist activity include: 1) a basic N-terminal amino group; 2) hydrophobic contributions from Phe^1, Val^2, and Ile^4; and 3) π-electron density and hydrogen bond donation from the Tyr^7 side chain.

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Induction of somatostatin and vasoactive intestinal peptide genes expression in the central nervous system of the adult rat by amino acids

Previous data have shown the neurotrophic effect of FACE a well defined amino acid mixture, on cortical and hypothalamic cells in culture. As amino acids interact with the blood brain barrier, the present study was performed to investigate the secretion and gene expression of the neuropeptides, somatostatin (SRIF) and vasoactive intestinal peptide (VIP), following subcutaneous FACE administration to adult male rats. The immunoreactive-SRIF (IR-SRIF) content in cerebro-cortical and hypothalamic tissue extracts was increased with 10^{-5} M FACE in a dose-response manner. Immunoreactive-VIP (IR-VIP) content in cor-

tical extracts was significantly increased with 10^{-5} M FACE. In the hypothalamus, there was a significant increase in IR-VIP using 10^{-5} M and no effect with 10^{-3} M. Somatostatin and VIP mRNA levels were increased in a dose-response manner in cerebrocortical tissues. Specificity of the regulatory activity of FACE on VIP was indicated by the lack of FACE effect on IR-VIP content in the anterior pituitary. Perhaps, due to the increased IR-SRIF in the hypothalamus, immunoreactive-GH (IR-GH) levels in the anterior pitruitary extracts tended to increase. Consequently there was a significant IR-GH decrease in peripheral blood after the administration of 10^{-3} M FACE. The present results demonstrate that the aminoacid mixture, FACE, has a powerful peptidergic effect on cortical SRIF and VIP secretory neurons in the intact animal, which actives the peptides content and the expression of their genes.

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α-Methylcysteine and its peptide chemistry

α-Methylcysteine (MeC) belongs to the family of α,α-disubstituted amino acids which offer an useful addition to the arsenal of peptide chemists. Interest in these amino acids is stimulated by their usefulness to produce restraints on conformational freedom of a peptide chain and by their potential to furnish peptides resistant to enzymatic attack and highly specific enzyme inhibitors.

We report here the absolute configuration of α-methylcysteine, N-, C-, S- protection results, coupling procedures serviceable for overcoming synthetic difficulties arising from steric hindrance, and examples of incorporation of α-methylcysteine into active peptides.

The resolution of the racemic MeC(Bzl)-OH was efficiently accomplished by crystallization of the salt of Boc-MeC(Bzl)-OH with quinine. Enzymatic approach has no preparative value because of rapid enzyme inactivation. Absolute configuration of (+)-MeC(Bzl)-OH was determined via four step chemical correlation with R(−)-α-methylserine. N-protected derivatives are accessible in moderate yield by routine methods; however a longer time of reaction and an excess of reagent is required. There are no restrictions as to the coupling methods which may be used to form peptide bond between the carboxyl group of N-protected MeC and amino group of protein amino acids. Coupling with sterically hindered amino acids was performed efficiently using BOP or TBTU reagent. The use of MeC in the synthesis of biologically active peptides is illustrated by the preparation of [MeC] glutathione, MeC-analogue of immunomodulator bucillamine, and by conformationally restricted MeC-analogues of angiotensin containing disulfide bridge.

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Covalent methionine enrichment of peptides for nutritional purposes

The biological value of food proteins is greatly influenced by the ratio of their essential amino acids. Besides the knowledge of the demand for the quantity and composition of amino acids, it is equally important to know whether these amino acids can be optimally utilized as free amino acids, in the form of peptides or as proteins. According to the current understanding of the science of nutrition, the biological utilization of mixtures of free amino acids is much less effective than that of amino acids in peptide chains.

The ability of proteinases to catalyze peptide bond synthesis has a historical background and has also a great recent interest.

An enzymatic technique called EPM (enzymatic peptide modification) has been elaborated for designed modification of proteins and peptides. In our recent study we apply the EPM reaction to have the following products of special nutritional character:

- methionine enriched products in order to increase the content of the methylating agent in the modified protein chains
- special amino acid (methionine) enriched peptides for improvement of the biological value
- proteins and peptides of reduced allergenic character. The fact that the allergenic character of proteins could be decreased by this enzymatic method is due very probably to the following factors: (i) transpeptidation which resulted in changes of covalent bonds within the peptide chains; (ii) amino acid enrichment of the modified peptides which leads to a favourable change in the structure of the proteins investigated.

Synthesis and Biosynthesis

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Partial synthesis of new analogues of the peptido-lactone Virginiamycine S_1, modified in the fifth and/or sixth position; ([Xxx^5]-VS_1 with Xxx = Ala, Asp, Asn and Lys and [Ala^4, Gly^6]-VS_1)

We achieved the reconstruction of VS_1-analogues containing a substitute for the fifth residue, y-oxo-Pip (Pip = pipecolic acid), starting from VS_1-pentapeptide (VS5P) the latter being prepared by a two-step degradation process of the native antibiotic VS_1 (Fig. 1). Protecting groups during the procedure were chosen in order to realize a minimal number of steps. Most of these gave excellent yields, including final cyclization between the fourth and fifth residue. In total, four analogues were synthesized with Ala, Asp, Asn and Lys replacing y-oxo-Pip. Among these, [Lys^5(Tfa salt)]-VS_1 is water-soluble, which is an important characteristic for eventual application of VS_1 as a pharmaceutical agent. In the proposed reaction sequence, we made sure that residues 4 (MePhe) and 6 (Phg) became partially epimerised. We therefore obtained each time after cyclization a total of four epimers that have been separated by preparative TLC, obtaining each a potential biological activity. The chiral identity of the final residues was realized by GC (Chirasil Val^R-III) on the total

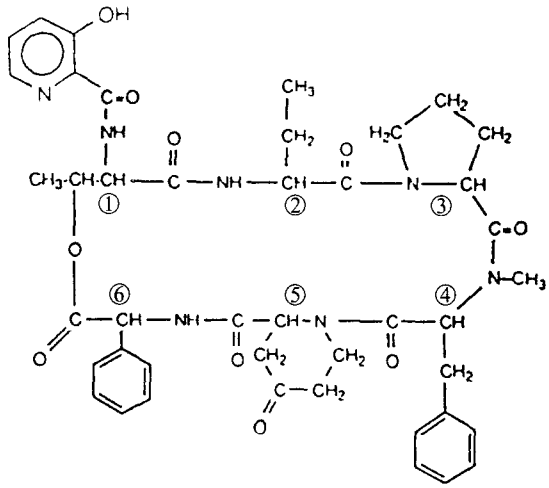

Fig. 1.

hydrolysates. Finally, a short racemization suppressing study was performed to synthesize one desired epimer with a maximum yield.

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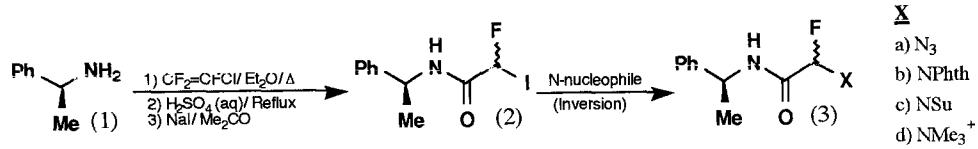
Use of the α-methylbenzylamine auxiliary in the asymmetric synthesis of α-fluoroglycine derivatives and analogues of pipecolic acid

α-Methylbenzylamine (1) is an excellent source of nitrogen for the asymmetric synthesis of amino acid; it is a cheap optically active starting material, available as either the (R)- or (S)-enantiomer, and possessing the chiral 1-phenylethl auxiliary that can be cleaved under mild conditions. Due to these attractive features, we have utilised α-methylbenzylamine as the chiral nitrogen source for research into the asymmetric synthesis of α-fluoroglycine derivatives and of analogues of pipecolic acid.

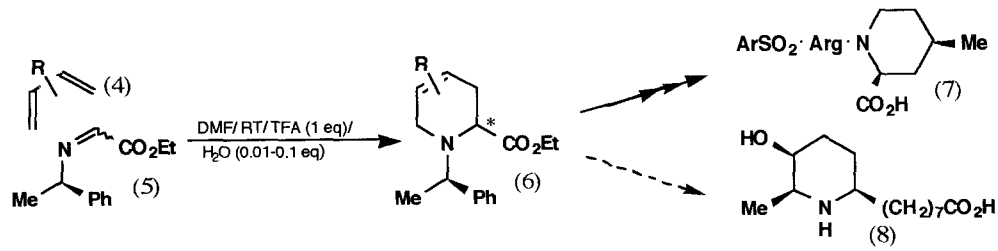
1) *α-Fluoroglycine derivatives.* It is unlikely that free α-fluoroglycine will ever be prepared, as the nitrogen lone pair is able to spontaneously displace fluoride ion, leading to decomposition of the amino acid. But when the nitrogen lone pair is involved in conjugation, then the resulting α-fluoroglycine derivatives should be moderately stable. This would be the case if the α-fluorinated residue were part of peptide, and such peptides should have intriguing biological properties as compared with the non-fluorinated counterparts – modified peptide bond properties (H-bonding characteristics and stability to hydrolysis), alterred electron density and polarisation, and the possibility of acting as suicide substrates.

Our method of gaining access to α-fluoroglycine derivatives is shown in Scheme 1. In this case, the 1-phenylethyl auxiliary fails to cause any asymmetric induction, but leads to diastereoisomers that are separable by flash chromatography. Displacement with nitrogen nucleophiles leads directly to α-fluoroglycine derivatives with high stereocontrol, and the auxiliary again facilitates removal of traces of the unwanted stereoisomers. The α-fluorobetaine (3d) is of special importance as, after hydrolytic removal of the auxiliary, it yields the first example of a "free" α-fluorinated α-amino acid zwitterion. We will also present our preliminary results on the incorporation of α-fluorinated residues into peptides.

2) *Analogues of pipecolic acid derivatives.* Pipecolic acid derivatives are of biological importance both as modified proline residues in peptides, and because of their intrinsic biological activity – e.g. carpamic acid (8). We recently developed an extremely rapid and versatile entry to the pipecolic acids using the aza-Diels-Alder reaction (see Scheme 2). Using this approach, high asymmetric induction is often observed (up to 93% d.e.), and the reaction also displays excellent diastereo- and regio-control within the piperidine ring. We also demonstrated that this reaction could be utilised in an extremely short and efficient asymmetric synthesis of the MQPA dipeptide thrombin inhibitor (7). We will also present our results on the application of this chemistry to the synthesis of carpamic acid (8).



Scheme 1



Scheme 2

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Large-scale production of chiral amino acid derivatives-useful for asymmetric synthesis

As pharmaceutically active substances are more and more produced in optically pure form, there is an increasing demand to obtain appropriate chiral auxiliaries for asymmetric synthesis in sufficient quality and quantity. Therefore, we developed technical syntheses for a variety of interesting and useful amino-acid-based chiral intermediates.

One example are chiral imidazolidinones like Boc-BMI introduced by Seebach et al. for the synthesis of optically active amino acids. First, recemic BMI, obtained by condensation of glycine methylamide with pivalic aldehyde and subsequent acidic cyclization, is isolated as crystalline HCl-salt. This allows efficient purification of this intermediate which is important for the following cleavage of the racemic mixture with mandelic acid in acetone. After N-Protection with Boc_2O, optically active Boc-BMI is obtained in fine, colourless crystals of high chemical and optical purity.

According to an alternate method developed by Schöllkopf et al., bislactimethers derived from diketopiperazines are alkylated with high diastereoselectivity to yield optically active amino acids after hydrolysis. For the most important diketopiperazines, cyclo-[Gly-Val] (L and D) and cyclo-[Gly-Tle] (L), technical production procedures were developed.

Finally, chiral oxazolidinones as described by Evans et al. are valuable synthetic auxiliaries for the asymmetric synthesis of a wide range of differently substituted carboxylic acids. Synthesis of these oxazolidinones starts from amino acids which are reduced to the corresponding amino alcohols followed by cyclization with carbonic acid derivatives.

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One pot synthesis of dipeptides by oxidative coupling method with dimethyldioxirane

Introduction. To reduce tedious and separate deprotection steps of amino and carboxyl protecting groups in peptide synthesis, we have devised a new convenient method. If we use formyl group as an amino protection group and methylthiophenyl (MTP) group as a carboxy protection group, oxidation reaction with dimethyldioxirane (DMDO) can remove the formyl group and activate MTP ester at the same time, leading to a peptide bond formation in one pot reaction.

Results and discussion. We have prepared some N-formyl amino acid derivatives and N-protected amino acid 4-methylthiophenyl (MTP) esters in order to perform one pot dipeptide synthesis by oxidative coupling method with dimethyldioxirane (DMDO) (0.0089–0.044 M). With this oxidizing agent, N-formyl group can be removed oxidatively probably via carbamic acid intermediate. At the same time, MTP ester group of another amino acid can be easily converted to 4-methylsulfonylphenyl (MSO_2P) ester group which is an active ester. As soon as free amino groups are released from the N-formyl protecting group, it will attack the active ester to form a peptide bond.

Scheme 1 presents a plausible mechanism for this one pot peptide condensation. According to this strategy, we prepared several peptides with relatively high yields. It is noticeable that the N-formyl amino acids or peptides which had free carboxylic groups at the α-position seemed to resist the DMDO oxidation, while all the MTP esters were readily oxidized into active esters. Moreover, when N-formylamino acids derivatives were treated alone by DMDO in the absence of another amino acid MTP esters, deformylation can not be properly controlled because of over-oxidation problems.

In conclusion, peptide fragments can be made conveniently from N-formyl amino acids (or peptides) and amino acid (or peptide) MTP esters by oxidation with DMDO. To optimize the reaction conditions and prove the detail reaction mechanism, more study is needed.

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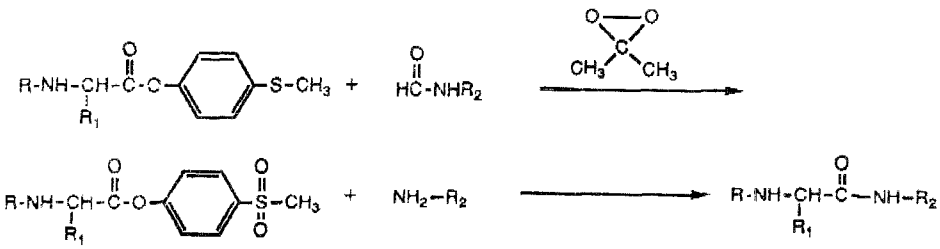
Chiral complexes for amino acid synthesis

Aminophosphinephosphinite-Rh-complexes derived from Propranolol analogues were synthesized. They are catalyzing with high activity and selectivity the asymmetric hydrogenation of derivatives amino acrylic acids to amino acids. By this approach unusual amino acids with different substitutents can be synthesized in a high optical purity. The synthesis of the ligands and the chiral catalysts as well as their application in amino acid preparation is demonstrated.



Scheme 1

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cGMP Formation in response to excitatory amino acids in cultured rat striatal neurons

In the brain, excitatory amino acids (EAAs), such as glutamate, play an important role in neurotransmission, and appear to mediate phenomena such as synaptogenesis and synaptic plasticity. EAA receptors can be divided in two groups: metabotropic and ionotropic, based on their coupling to a G protein or to an ion channel respectively. The ionotropic receptor can be furtherly subdivided in NMDA and NON-NMDA type, based on their affinity for N-methyl-D-aspartate. Several second messengers, such as arachidonic acid and inositol phospholipid metabolites, have been described to be coupled to the activation of selective EAA receptor subtypes. Thus, the development of a responsiveness to EAAs in striatal neurons is of interest for understanding the process of synaptogenesis in the striatum. Here, we present data on the cGMP formation following the activation of EAA receptors in striatal neurons in primary culture. Primary cultures of striatal neurons were prepared from 15–20 day-old rat embryos, and maintained for more than 20 days in culture (DIC). Intracellular cGMP levels after EAA receptor stimulation were measured at different DIC. Maximal stimulation (3–4 times over basal levels) was achieved at 13–17 DIC. Maximal stimulation of cGMP formation by glutamate was obtained at 50 μM when Mg^{++} was absent, and at 1 mM when Mg^{++} (1 mM) was present. N-methyl-D-aspartate (NMDA, 10 μM) produced a maximal stimulation of cGMP formation in the absence of extracellular Mg^{++}. On the other hand, 1 mM NMDA was required in order to obtain a cGMP stimulation of the same magnitude, when Mg^{++} was present. Kainate, an agonist of the ionotropic NON-NMDA receptor, induced a dose-dependent elevation of cGMP levels, maximal at 100 μM (3–4 times over basal levels). Maximal stimulation of cGMP formation by quisqualate, an agonist of both ionotropic and metabotropic NON-NMDA receptors, was limited to 1.5–2 times over basal levels and was achieved at 5 μM. The NMDA receptor antagonist amino-phosphonovalerate (APV, 1 mM) blocked the cGMP response to NMDA while it did not affect the response to kainate. cGMP formation by either kainate or quisqualate was blocked by the ionotropic NON-NMDA receptor antagonist CNQX (50 μM). The time course of cGMP formation induced by EAAs reached a maximum from 30 sec. to 2 min., after the addition of the agonist, and was dependent upon extracellular calcium. cGMP formation was not associated with the formation of cAMP. Evidence suggesting a cGMP formation independent from that of other second messengers, will be discussed.

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Modulation of glucose consumption and excitotoxicity by endogenous excitatory amino acids in neuronal cultures

Cerebellar neurons in primary culture are undergoing morphological, ultrastructural, biochemical and electrophysiological differentiation. No data are available on whether the achievement of full differentiation may involve changes in the cellular energy requirements. On the other hand, it is known that toxicity by excitatory amino acids (EAAs) in fully differentiated cultured cerebellar neurons is dependent upon cellular energy charge. Here we report that glucose consumption presented two linear phases during days in culture (DIC), changing from ~ 1 μmol glucose/10^6 cells $\times$ DIC from 3 to 12–15 DIC (phase A) to ~ 2 μmol glucose/10^6 cells $\times$ DIC from 12–15 to 26 DIC (phase B). The number of neurons in culture decreased by $\sim 40\%$ from 0 to 3 DIC and then remained constant at $\sim 10^6$ neurons/35 mm dish. The number of non neuronal cell was limited to $\sim 3\%$ of total by the addition of ARA-C (10 μM) at 1 DIC. Protein content doubled from 3 to 12 DIC, and then remained constant at ~ 200 μg/10^6 cells thereafter. Thus, when glucose consumption was calculated with respect to protein content, the ratio between phase B and phase A was ~ 4. We investigated whether phase B of glucose consumption could be dependent upon generic energy-dependent enzimatic activities, such as NAD(P)H-dependent reductase, or could be specifically related to glutamatergic neurotransmission. NAD(P)H-dependent reductase activity, as measured by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan, increased from ~ 400 nmol formazan/mg protein at 3 DIC to ~ 800 nmol formazan/mg protein at 12 DIC and remained constant thereafter, thus excluding a correlation between phase B of glucose consumption and reductase activity. On the other hand, antagonists of the NMDA-type of EAA receptors, such as aminophosphonovaleric acid (APV) and MK-801, significatively reduced phase B of glucose consumption, while they did not affect phase A, indicating a role for endogenous EAAs in the control of phase B. According to this hypothesis, the glutamate reuptake inhibitor threohydroxy-aspartate (THA) significatively increased glucose consumption above the value of phase B, while increasing endogenous EAAs concentration in the growth medium until causing excitotoxicity. Similar results were obtained upon stimulation of endogenous EAAs release. We suggest that the appearance of phase B of glucose consumption in cerebellar neurons in primary culture may be specifically related to the energetic requirement of fully developed glutamatergic neurotransmission.

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Cloning and expression of glutamate receptor subunit isoforms during CNS development

Fast excitatory synaptic transmission in the CNS is predominantly mediated by ligand-gated glutamate receptors (GluRs). The role of these receptors in development, neurodegenerative disease and neurotoxicity is widely accepted and has recently captured considerable attention. We are using molecular biology, combined with pharmacology and electrophysiology, to investigate the molecular diversity of native receptors found in dorsal root ganglia (DRG) neurons and cerebellar granule cells, in order to assess the contribution of subunit heterogeneity on the diverse receptor properties expressed in these cells. Northern blot and PCR analysis of the DRGs show that, although all the non-NMDA receptor subunits are expressed, the kainate-preferring

subunits (particularly GluR5, KA-2 and KA-1) are predominant. GluR5 transcripts are expressed in the DRGs at about 5-fold greater levels than other kainate-preferring subunits, and about 10-fold greater than AMPA-preferring subunits. GluR5 is expressed at 2–3-fold higher levels in the DRG than the brain. Reconstitution experiments using the cloned sequences in oocytes and transfected cells, corresponding to the predominant subunits expressed in DRGs, show similar pharmacological characteristics seen in neurons. Thus, any combinations made with GluR5, KA-1 and KA-2 give desensitizing responses to kainate that are made non-desensitizing in presence of ConA, but not cyclothiazide, differentiating these receptors from those typically studied in hippocampal neurons.

Cerebellar granule cells were found to express predominantly GluR 2, 4 and KA-2. Transcripts derived from the GluR4 gene were found to be differentially processed in regions coding for the C-terminus, between putative transmembrane III and IV, and in the region residing upstream of the first transmembrane domain. Splicing of the latter, results in a 3.0 kb polyadenylated transcript that was cloned from a cDNA library. The cDNA, designated GluR4s (the "s" stands for short), codes for a putative subunit lacking the transmembrane domains. GluR4s mRNAs, in contrast to other GluR transcripts that are expressed in granule cells, astrocytes and Bergmann glia, are predominantly confined to granule cell neurons. Furthermore, the expression of GluR4s mRNAs accumulate during neuronal development. The function of GluR4s awaits elucidation. The contribution of GluR4s, as well as different GluR isoforms, on granule cell neurotoxicity is under investigation.

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Modulation of glutamate receptor function in cerebellar granule cells cultured in vitro

Cerebellar granule cells constitute an excellent model system to analyze the conditions that modulate the expression/and or function of glutamate receptors and their involvement in excitotoxicity. We have recently reported that when these neurons are grown in the presence of a serum protein complex named NOAC (neurite outgrowth adhesion complex) they form an intense network of neurites, survive for long periods in vitro, express several phenotypic markers typical of neurons, but exhibit a marked resistance to the otherwise lethal action of excitatory aminoacids (EAAs). We found that when these neurons are grown in the presence of NOAC plus human recombinant IGF-I (25 ng/ml) they express functionally active glutamate receptors and become fully sensitive to the toxic action of glutamate and other EAAs. The action of IGF-I is concentration (half maximal effect at 1.5–3.0 ng/ml), time dependent (half maximal effect 2–3 days of culture) and is rapidly reversible ($t1/2 = 60$ min.) after removal of IGF-I. The "sensitizing" action of IGF-I is accompanied by the appearance of glutamate-activated Ca++ permeable channels. on the basis of these findings and of other experiments to be reported we postulate that the constitutive phenotype of cerebellar granule cells is EAA− (resistant to glutamate and other EAAs) and becomes EAA+ (sensitive to glutamate) in the presence of IGF-I and possibly of other polypeptides present in serum and in other biological fluids. The initial characterization of such polypeptide(s) will be presented and discussed on the ligth of their possible relevance to physiological or pathological conditions.

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N-Methyl-D-aspartate receptor-mediated neuroprotection in cerebellar granule cells requires new RNA and protein synthesis

Cultured cerebellar granule cells are glutamatergic neurons which express all of the glutamate receptor subtypes. Previous studies have shown that cultured cerebellar granule cells are susceptible to the neurotoxic effects of the excitotoxin glutamate and the chemical neurotoxin, 1-methyl-4-phenylpyridinium (MPP^+). Although the toxicity of glutamate and MPP^+ are time- and concentration-dependent in cultured cerebellar granule cells, the mechanisms of toxicity are different. Whereas glutamate mediates its toxicity by the activation of N-methyl-D-aspartate (NMDA) receptors, MPP^+ kills neurons by intracellular uptake into vulnerable neurons. Paradoxically, preincubation of cultured granule cell neurons with subtoxic concentrations of NMDA or glutamate markedly antagonizes the neurotoxicity from subsequent exposure to toxic concentrations of either MPP^+ or glutamate. The neuroprotective effects of NMDA and glutamate against MPP^+ toxicity are observed at concentrations as low as 1 μM, blocked by specific NMDA receptor antagonists and require at least 30 min to fully develop. Preexposure of the neurons to subtoxic concentrations of NMDA also resulted in significant protection against toxic glutamate concentrations (50–1000 μM). Moreover, NMDA-receptor mediated neuroprotection is prevented by the RNA synthesis inhibitor, actinomycin D, or the protein synthesis inhibitor cycloheximide. Thus, activation of NMDA receptors by glutamate results in either neurotoxicity or neuroprotection, depending on the glutamate concentration and apparent degree of receptor stimulation. NMDA receptor-mediated neuroprotection in these neurons requires new RNA and protein synthesis and appears to be mediated by the expression of a neuroprotective protein(s). Taken together, these data demonstrate the presence of an active NMDA receptor-mediated and transcriptionally-directed neuroprotective mechanism in cerebellar granule cells.

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Pharmacology of domoic acid and related toxic amino acids in food

The pharmacology of domoic acid (DOM) was first described in Daigo (1958) as a naturally-occurring constituent of the red algae *Chondria armata*. Subsequent *in vitro* studies in the late 1970s and early 1980s led to the classification of DOM as a highly potent excitatory amino acid (EAA) agonist acting primarily at non-NMDA (AMPA/kainate) receptors. Interest in the pharmacology of DOM increased significantly in 1987 when the compound appeared as a contaminant in commercial blue mussels (*Mytilus edulis*) harvested from eastern Prince Edward Island, Canada. Human toxicity manifested in a variety of symptoms ranging from gastrointestinal distress to loss of motor coordination, seizures, memory loss and death. Recently, DOM has also appeared as a contaminant in razor clams and other crustaceans harvested from the western United States. Consequently, most of the recent work on DOM has concentrated on the pharmacology of the EAA *in vivo*, in an attempt to devise rational therapeutic strategies for dealing with subsequent outbreaks of DOM poisoning. This work has focused along two main lines of investigation; pharmacokinetics and neurotoxicology.

The pharmacokinetics of DOM have been described in a variety of rodent and primate models. The oral bioavailability of DOM is extremely low in all species examined to date. Following either intraperitoneal or intravascular administration in rats DOM distributes rapidly with a limited volume of distribution (Vd) and is rapidly eliminated, mainly via the kidney. Penetration of both the blood-brain barrier and the placental barrier in the rat is marginal.

Despite relatively poor penetration into the CNS, DOM is a potent neurotoxin when administered to monkeys, rats and mice (see Strain and Tasker, Neuroscience 44: 343, 1991). Neuronal damage occurs in a number of brain regions, particularly the CA3 hippocampal subfield, but the overall pattern is more selective than that seen with kainic acid. Recent evidence from our laboratory indicates that much of the toxicity of DOM can be attributed to a selective action at a receptor corresponding to the low affinity ^{3}H-kainic acid binding site, and can be antagonized by the recently described isanoxitime, NS-102 (Nielsen et al., Soc. Neurosci. 18: 86, 1992). In addition, DOM has been shown to selectively interact with a variety of other neurotransmitter systems.

Most recently, our laboratory has begun to investigate the actions of DOM at physiological, rather than pathological concentrations. Preliminary results indicate that DOM-sensitive systems are involved in a variety of important functions including normal learning and memory and spinal mechanisms of nociception. In addition to its established roles as a neurotoxin and as a toxic contaminant of food, DOM is becoming an increasingly useful probe for investigating selective aspects of AMPA/kainate receptor function.

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Neurochemistry

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Positive modulation of NMDA receptors as a therapeutic approach for symptomatological treatment in dementia

The cellular mechanisms of learning and memory have begun to be specified. It is widely believed that a persistent enhancement of synaptic responses, long-term potentiation (LTP), is an underlying mechanisms of learning and memory. At many synapses, the induction of LTP requires activation of the N-methyl-D-aspartate (NMDA) receptor complex. In particular, LTP at the Shaffer collateral/commissural-CA1 synapse in the hippocampus is dependent on NMDA receptor complex activation. The hippocampus is well-known to play a role in the kinds of learning and memory which are impaired in aging and in Alzheimer's disease.

In addition to binding by an NMDA agonist, activation of the NMDA receptor complex also requires activation of a glycine site on the complex. Therefore, compounds which activate the NMDA receptor-associated glycine site should increase the probability of inducing LTP and increase learning. D-cycloserine, a known tuberculostatic agent, is a potent and bioavailable partial agonist of this glycine site exhibiting an efficacy of 40–70% relative to glycine.

In animal studies, D-cycloserine has been shown by several laboratories to improve learning and/or memory consolidation and to improve memory retrieval. These effects have been seen in three species. The magnitude of the effect appears to depend on the assay used. In most cases, the dose-response relationship is a U-shaped function. Unexpectedly, at high doses, D-cycloserine produces behavioral effects similar to those produced by antagonists of the NMDA receptor complex. This effect is probably not related to the partial agonist property of D-cycloserine. In addition, in some studies the effects of D-cycloserine disappear following repeated administration. These unexpected effects might be explained in terms of results from *in vitro* studies showing that inappropriately timed activation of the NMDA receptor complex can block LTP.

Acute administration of DCS in humans can overcome learning and memory impairments induced by scopolamine. DCS has also been examined during chronic administration in humans classified as having age-associated memory impairment (AAMI) or Alzheimer's disease. Although some improvements were noted in the AAMI patients early on the trials, no consistent or lasting improvements were noted. This result may be similar to the effects of repeated administration seen in some animal studies.

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Characterisation of learning and memory deficits following NMDA receptor antagonism

Several lines of evidence suggest that glutamatergic neurotransmission, mediated through NMDA receptors, is involved in the processes of learning and memory. NMDA antagonists have been reported to impair learning and memory in rats in several different tasks. These drugs also induce profound motor deficits even at low doses, and this may interfere with performance of learning and memory tasks where motor skills are required. Reversal learning in a Y-maze can obviate this difficulty since learning is assessed by response accuracy rather than response latency, and performance in reversal can be compared to acquisition.

In the present study we have examined the effects of the NMDA antagonist, dizocilpine on acquisition and reversal of spatial and visual discrimination tasks in a Y-maze. Male Lister hooded rats maintained at 90% of their free-feeding bodyweight were used in all experiments. They were tested individually in a Y-maze of three equal arms; length 60 cm, width 11.5 cm, height 25 cm. Correct responses were reinforced with food pellets.

Dizocilpine (0.1 $mgkg^{-1}$) produced a distinct behavioural syndrome which included hyperactivity. Higher doses resulted in decreased activity and ataxia. Treatment with dizocilpine (0.075 $mgkg^{-1}$) was without effect on the acquisition of a spatial task; however on reversal dizocilpine-treated rats took significantly more trials to reach criterion (45.9 $\pm$ 7) than controls (17.9 $\pm$ 1.4, $p < 0.01$). On the visual discrimination task dizocilpine impaired both acquisition (saline 37.1 $\pm$ 7.8; dizocilpine 84.0 $\pm$ 20.6) and reversal (saline 107 $\pm$ 9; dizocilpine 172 $\pm$ 17).

These experiments demonstrate that dizocilpine produces a learning impairment. The lack of impairment on spatial acquisition argues against a global performance deficit. Other studies,

mainly using the water maze, have demonstrated impairments in spatial but not visual learning following treatment with NMDA antagonists. The results of the present experiments demonstrate effects on both spatial and visual tasks, although more profound on the former.

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Glutamate, transcription factors and learning

Transcription factors (TF) are proteins involved in control of gene expression. In recent years several TF have been shown to be inducible in the brain and cultured brain cells by a plethora of different stimuli. The common link between all those phenomena seems to be an acquisition of new, long lasting features by stimulated cells in vivo and in vitro. Long term memory formation may also be described as a process involving long term neuronal changes, namely reorganization of efficacy of synaptic connections. Interestingly, activation of L-glutamate receptors are known to be involved both in long term memory formation and elevation of AP-1 transcription factor. AP-1 is a protein dimer made of members of Fos and Jun families.

In our studies we have found that increased DNA binding activity of AP-1 follows stimulation of L-glu receptors in cultured neurons and brain regions in vivo. In cultured neurons derived from rat fetal cerebral hemispheres both NMDA and non-NMDA receptors were involved. In the brain AP-1 elevation was observed after epileptogenic treatment with either pentylenetetrazole or kainate.

Elevated expression of c-fos gene, encoding a component of AP-1 was found after high frequency stimulation of perforant path, leading to long lasting long term potentiation as well as after different forms of behavioral training including acquisition of two-way active avoidance reaction and learning of copulatory behavior in sexually naive male rats.

These results suggest that L-glutamate receptor driven activation of AP-1 transcription factor may be at the bases of long term changes in neuronal functioning, including those underlying long term memory formation.

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Functional interactions between glutamate and dopamine in the basal ganglia

The striatum, primary afferent structure of the basal ganglia, receives excitatory amino acid (EAA) projections, presumably glutamatergic (GLU), from the cortex and a dopaminergic (DA) from the mesencephalon. Blocking EAA transmission at the N-methyl-D-aspartate (NMDA) receptor subtype has been shown to produce behavioral activations similar to those induced by an enhancement of DA transmission thus revealing a functional antagonism between GLU and DA systems. To further investigate the DA-NMDA interactions within the ventral and dorsal part of the striatum, the effects of bilateral intrastriatal microinjections of the competitive NMDA receptor antagonist AP-5 were studied in a spontaneous and a conditioned motor task in the rat.

AP-5 (2, 4 and 10 $\mu g/\mu l$/side) in the ventral striatum induced a dose-dependent increase in locomotor activity similar to that induced by a ventral striatal DA injection. Blockade of DA receptors with specific D1 and D2 antagonists reversed the locomotor hyperactivity induced by 10 μg APV. In a second experiment, animals were trained to depress and hold a lever until the presentation of a visual conditioned stimulus (CS) and then to release it with a reaction time (RT) of less than 600 ms for food reinforcement. AP-5 (5 $\mu g/\mu l$/side) in the dorsal striatum induced a behavioral motor activation expressed by an increased number of anticipatory responses (release of the lever before the CS) and shortened RTs, as previously observed with a DA injection in the same location. The stimulant effects of AP-5 were not potentiated by a concomitant intrastriatal injection of DA (2.5 $\mu g/\mu l$/side) and could be reversed by the D2 DA receptor antagonist, raclopride (50 μg/kg) injected systemically. At low dose (0.5 $\mu g/\mu l$/side). AP-5 did not affect RT performance, but reduced the activation effect of DA and dramatically potentiated the increase in the number of delayed responses induced by raclopride. A functional antagonism between EAA-DA systems within the striatum was only observed after a total blockade of NMDA receptors with a high dose of AP-5 suggesting that more complex mechanisms might underly EAA-DA interactions.

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Endothelin-1 increases blood pressure in rat via selective involvement of the NMDA receptors: researches in the PAG area

Endothelin-1 (ET-1) is a potent and long-lasting vasoconstrictor peptide which may act as a neurotransmitter within the central nervous system. The present study was designed to examine the possible relationship, in the dorsolateral PAG area, between ET-1 and the glutamergic system, that is well known be present in this area too, in the regulation of the cardiovascular function in freely moving rats. Male Sprague Dawley rats (250–300 g) were used in this study. Two days before the experiments a stainless steel guide cannula for the intracerebral microinjections was inplanted in the dorsolateral PAG area of rats anaesthetized with ketamine. On the day of the experiment a catheter was inserted into the femoral artery under 2% halothane anaesthesia for the mesurement of blood pressure (BP) by a pressure transducer connected to a Hellige polygraph. Microinjections of ET-1 (0.1–1–10 pmol/rat) in the dorsolateral side of the PAG area significantly ($p < 0.05$) and immediately increased BP (respectively of 14 ± 2, 48 ± 5, 55 ± 4 mmHg; $n = 5$). The pretreatment with 2-APV (1 μg), a selective NMDA receptor antagonist, but not with CNQX (0.01 μg/rat), a selective non-NMDA receptor antagonist, reduced (-90%; $n = 5$; $p < 0.01$) the ET-1 induced cardiovascular changes. Like 2-APV, the non competitive antagonist of the NMDA receptors, MK-801 (1 mg/kg *ip*), reduced (-100%; $n = 4$; $p < 0.01$) the hypertension induced by ET-1. Finally, the cardiovascular changes induced by ET-1 were also antagonized by systemic administration of prazosin (1 μg/kg *ip*), propranolol (0.1 mg/kg *ip*) and by reserpine (5 mg/kg by subcutaneous route 24 h before ET-1). This study shows the first evidence *in vivo* that at the level of PAG area the NMDA glutamergic subtype receptors may have an important role in the ET-1 induced pressor effects. In particular, assuming that the endogenous excitatory amino acids stimulate the release of catecholamines we speculate that the primary effect of ET-1 might be a Ca^{2+}-dependent release of glutamate that later on, mainly by means of the NMDA subtype receptors' activation, facilitates the monoaminergic pathways.

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Interaction between striatal excitatory amino acid and GABA receptors in the turning behaviour of rats

Rotational behavior is one of the most extensively used models of the striatal dopaminergic activity. Following a unilateral lesion of the nigrostriatal dopaminergic pathway, agonists of dopamine receptors induce contralateral turning. The dopamine-dependent rotational activity is primarily mediated by striatofugal GABAergic efferents which project to the substantia nigra pars reticulata (SNR). It is postulated that the activity of the GABAergic striatonigral pathway is under a tonic inhibitory influence of striatal GABAergic receptors. It was reported that intrastriatal muscimol or baclofen injections led to an ipsiversive postural response, whereas intrastriatal picrotoxin injections induced contralateral turning. Moreover, it was demonstrated that an increase in the GABAergic transmission in the SNR at the level of a striatonigral input induced vigorious contralateral rotations. It has also been postulated that striatonigral neurons receive a glutamatergic monosynaptic input from the sensorimotor cortex. Within the striatum, glutamate acts on different receptors, such as NMDA, kainate, AMPA and metabotropic, whose density in this structure is the highest in the basal ganglia. Binding studies have shown that part of these receptors are localized on striatonigral projection neurons. It has also been reported that intrastriatal injections of NMDA, kainic, glutamic, or quisqualic acid and an agonist of metabotropic receptors (trans-ACPD) induce contralateral rotations. The latter result may suggest that, like dopamine receptors, excitatory amino acids stimulate the GABAergic striatonigral pathway. Therefore it is not unlikely that drugs acting on excitatory amino acid receptors offer an alternative therapy of Parkinson's disease. The aim of the present study was to examine the interaction between excitatory amino acid receptors and GABAergic receptors in their influence on striatonigral neurons. To this end, drugs acting on those receptors were injected into the region wherefrom the striatonigral pathway originated and the rotational behavior of rats was evaluated.

NMDA (500 ng/0.5 μl), kainic acid (50 ng/0.5 μl) and AMPA (1000 ng/0.5 μl), injected into the above-mentioned region, induced contralateral head turns and rotations. Picrotoxin (250 ng/0.5 μl), injected 30 min before NMDA, kainic acid or AMPA significantly increased the number of the contralateral head turns and rotations induced by those excitatory amino acids.

The present results seem to suggest that (1) NMDA, kainate and AMPA receptors, together with GABAergic ones, are present on the same striatonigral neurons; (2) the contralateral behavior induced by agonists of the excitatory amino acid receptors appears to be a suitable model for studying functions of these receptors in the striatum.

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The role of striatal excitatory amino acid receptors in the turning behaviour of rats

Excitatory amino acid receptors have been postulated to play an important role in the motor behaviour of rats, as well as in Parkinson's disease in humans. It is suggested that antagonists of NMDA and non-NMDA receptors may be beneficial in Parkinson's disease because of their "antiparkinsonian" effect in models of this disease in animals. It has been found that at least part of the excitatory amino acid receptors which are responsible for these effects are located in the caudatus-putamen. Of the basal ganglia, the caudatus-putamen has the highest density of these receptors which are mainly present on both intrinsic and output striatal neurons. Two main striatal output pathways, a striatopallidal pathway and the striatonigral one, are GABAergic. The striopallidal pathway originates mainly in the anterior, and the strionigral pathway – in the intermediate ventrolateral part of the caudatus-putamen. However, the role of the excitatory amino acid receptors, located on cells of each of these output pathways, in the motor behaviour is unknown. The aim of the present study was to investigate the role of NMDA, AMPA and kainate receptors, present in the region of the striatonigral pathway output cells, in the rotational and asymmetric behaviour of rats, which is one of the commonly used animal models of Parkinson's disease. In this model, substances that can be useful as antiparkinsonian drugs induce contralateral rotations. The experiment was performed on male Wistar rats, implanted unilaterally and chronically with guide cannulae. We examined the influence of different drugs acting on the excitatory amino acid receptors, which were injected into the ventrolateral part of the intermediate region of the caudatus-putamen wherefrom the striatonigral pathway originates. Rotational behaviour was recorded either with an automatic rotometer or by direct observation. After the termination of the experiment, localization of the cannula tips within the caudatus-putamen was checked histologically in each rat. N-methyl-D-aspartate (100, 250 and 500 ng/0.5 μl), kainic acid (50 and 100 ng/0.5 μl) and AMPA (500 and 1000 ng/0.5 μl), injected into the ventrolateral part of the caudate-putamen, induced contralateral head turns and rotations. The antagonist of non-NMDA receptors DNQX, used in a dose of 1 μg/0.5 μl, antagonized the contralateral head turns and rotations induced by AMPA (1 μg/0.5 μl) or kainic acid (50 ng/0.5 μl).

The present results seem to suggest that stimulation of NMDA, kainate and AMPA receptors located on the striatonigral pathway might exert an antiparkinsonian effect. However, this conclusion is in strong contrast to common expectations if we bear in mind that antagonists of the excitatory amino acid receptors given systemically, seem to have beneficial effects in many animal models of Parkinson's disease.

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Galanin receptor ligands: design and synthesis of high-affinity antagonists

Galanin, a 29 amino acid long neuropeptide, first isolated from pig upper intestine, exerts a variety of biological effects. It inhibits the induced acetylcholine release in hippocampus, it stimulates growth hormone release, it stimulates feeding behaviour upon hypothalamic injection, it inhibits the glucose-induced insulin release and it has a biphasic effect on the spinal flexor reflex. We have synthesized a series of high affinity galanin receptor ligands. The N-terminal portion of galanin is important for biological activity as well as for high-affinity ligand binding. The shortest N-terminal galanin fragment with reasonably high affinity is galanin(1–12). IC_{50} values for endogenous galanin, galanin(1–16) and galanin(1–12) in rat hypothalamus are 1 nM, 8 nM and 1 μM, respectively, using ^{125}I-monoiodo-Tyr^{26}-porcine galanin as tracer ligand. The synthesis of chimeric peptides, consisting of N-terminal part of galanin, galanin(1–13) and C-terminal part of another biologically active peptide re-

sulted in high-affinity galanin receptor ligands having in some cases more than 10-fold higher affinity than endogenous ligand, galanin(1–29). Some of these peptides act as galanin receptor antagonists, blocking the effect of galanin in various biological systems. Furthermore, M40, a peptide, in which the N-terminal galanin(1–13) is extended by the peptide sequence, $(Pro)_2$-$(Ala\text{-}Leu)_2$-Ala, is found to be a galanin receptor antagonist reversing the effects of galanin in hippocampus and hypothalamus, but not in spinal cord and pancreas. In the basis of different effects of M40 and of other chimeric ligands in blocking the effects of galanin we have defined the putative galanin receptor subtypes.

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Are low affinity σ sites, the sites of anticonvulsant action, by inhibition of glutamate release?

It is now clear that σ binding sites are distinct from opioid sites and that they show preferential affinity for the (+)-isomers of certain opiates such as SKF-10047, cyclazocine and pentazocine. Also binding studies using σ and PCP (phencyclidine) selective ligands, indicate that the two sites represent distinct molecular entities, and that the PCP site is a component of the NMDA receptor-ionophore complex. Sigma binding sites occur in various limbic structures of the brain and currently the σ site is the object of intense research in numerous laboratories. There is evidence that multiple σ sites such as σ_1 and σ_2 may exist with different affinities for different σ ligands. Thus haloperidol and 1,3-di-o-tolylguanidine (DTG) exhibit high affinity for both σ_1 and σ_2 sites, but carbetapentane and dextromethorphan (DXM) have high nM affinity for the σ_1 sites and low μM affinity for the σ_2 sites, as also the (+)-isomers of pentazocine and SKF-10047, although their (−)-isomers demonstrate only low to moderate affinities for both σ sites. Availability of functional data on the role of σ sites is still very limited, although the high affinity of the antipsychotic drug haloperidol for the σ_1 sites may provide a target for a new class of antipsychotic drugs. However, the lack of receptor specificity of even the most selective of the available σ drugs leads to confusion as to which of the drug effects could be definitely attributed to σ site activation. In order to identify the functional correlates of σ receptor activation, one of the key requirements would be the development of σ ligands with improved selectivity and specificity which would help in understanding the σ site functions along with their potential for drug targeting. In this paper, we describe a new class of potent orally active anticonvulsant agents, the aminoalkylpyridines (AAPs), that are low μM affinity σ ligands with high σ selectivity. The AAPs weakly displace [^{3}H] DTG, a ligand highly specific for the σ receptor (50–60% at 10 μM), with a high degree of correlation between σ activity and anticonvulsant potency. They also inhibit glutamate release (70–80% at 50 μM), but fail to displace NMDA receptor ligands including [^{3}H] NMDA and [^{3}H] glutamate. These results strongly suggest that low affinity σ ligands may be anticonvulsant agents and low affinity σ sites may be sites of anticonvulsant action by inhibition of glutamate release. The anticonvulsant activity of DXM and other σ ligands also appears to be associated with their low affinity σ binding components. The low affinity σ specific AAPs may provide valuable tools to investigate low affinity σ sites.

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Behavioural pharmacology of dopamine and glutamate in the basal ganglia

The striatum receives prominent glutamatergic afferents from all neocortical areas and a dopaminergic projection from the mesencephalon. The cortical inputs are transmitted via a direct and an indirect striato-thalamic route back to the cortex. The behavioural relevance of the dopamine-glutamate balance in the basal ganglia is indicated by our experiments using systemic or local injections of dopamine (DA) or glutamate (GLU) (ant-)agonists. Intracerebral infusion of either kynurenic acid, a broad-spectrum glutamate antagonist, or APV, a selective antagonist of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors, into the striatum of rats produced a behavioural stimulation, e.g. locomotion and stereotyped sniffing. Striatal output neurons bearing NMDA receptors probably mediate these effects, because selective destruction of these neurons by striatal lesions with quinolinic acid produced similar effects dependent on the striatal site of the lesion. Thus striatal DA and NMDA receptors were suggested to mediate opposite behavioural effects. Indeed the non-competitive NMDA antagonist dizocilpine reversed haloperidol-induced catalepsy. Systemic administration of other non-competitive and competitive NMDA antagonists also induced locomotor stimulation and reversed neuroleptic-induced catalepsy. However, considerable differences exist between their profile of behavioural actions, potencies and neurochemical actions. In addition NMDA antagonists differentially reversed haloperidol-induced akinesia and bradykinesia in a reaction time task. Thus NMDA antagonists may provide a strategy for the treatment of Parkinson's disease (PD), but undesirable effects already seen in rodent models may limit their use. In contrast non-NMDA antagonists did not change spontaneous motor activity, failed to antagonize or even enhanced haloperidol-induced catalepsy and reversed the anticataleptic activity of dizocilpine. Thus non-NMDA antagonists act behaviourally suppressive in these models and a therapeutic value for PD of these compounds is thus uncertain at present.

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Binding and transport of Leucine-Enkephalin in isolated bovine brain microvessels

The presence, both in the central nervous system and in peripheral tissues, of several biologically active peptides raises the problem of their selective permeation across the blood-brain barrier. By investigating isolated rate brain microvessels – which are the anatomical counterpart of the BBB – Pardridge et al. (Endocrinology 109: 1138–1143, 1981) had concluded that Leucine-Enkephalin (Leu-Enk) was rapidly degraded and that no binding site or specific transport system for this neuropeptide existed in this *in vitro* system. By using an *in vivo* vascular brain perfusion method, Zlokovic et al. (J. Neurochem. 53: 1333–1340, 1989) have instead found evidence in favour of the existence of a progressive

saturable luminal uptake of Leu-Enk: the circulating neuropeptide appeared to cross the blood-brain barrier (BBB) in its native form, without any inhibition by large neutral amino acids, and no enkephalinase activity could be demonstrated on the luminal side of the BBB. The aim of the present study, which made use of an *in vitro* preparation of isolated bovine brain microvessels, was to identify the binding site(s) and to verify the subsequent capacity of Leu-Enk to be transported across the brain endothelial cell membranes. All the experiment were carried out in the presence of 9 μM Bestatine, a specific inhibitor of aminopeptidase activity. The results of the binding experiments, subjected to Scatchard analysis, revealed the presence of an upward concave curve in which the high affinity site(s) (δ receptors, as judged by competition with other ligands) was characterized by K_{D1} = 0.065 nM and B_{max1} = 66.6 pmol/mg protein, while the low affinity site(s) (probably μ receptors) has a K_{D2} = 7.93 nM and B_{max2} = 1118 pmol/mg protein. The time-course uptake of either labeled Leu-Enk or labeled (D-Ala^2-D-Leu)-Enk – a synthetic peptide, which is not substrate of brain enkephalinase and is mainly an agonist for δ receptors – indicated that the neuropeptides tested were transported inside the endothelial cells, as intact molecules, by a glucose- and Na^+-independent system, equilibrium being reached within approximately 20 min. The kinetic analysis showed the presence of a single saturable transport system, with $K_M = 480 \pm 11$ μM and $V_{max} = 140 \pm 8$ pmol/min/mg protein. In conclusion, the present study suggests the presence of specific Leu-Enk receptor/transport system(s) on the isolated brain microvessels, thus confirming the earlier *in vivo* studies on enkephalins permeation across the BBB.

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Evidence that nitric oxide is involved in the pathophysiology of seizures and neurodegeneration in rats

Nitric oxide (NO), a short-lived highly reactive raidcal species, has been suggested to function as neuronal messenger in the mammalian brain (Garthwaite et al., 1988, Nature, 336: 385–388; Moncada et al., 1989, Biochem. Pharmacol., 38: 1709–1715). Recently, it has been shown that L-NAME, an inhibitor of NO synthase, protects rats from seizures and related demage produced by the anticholinesterase agent, tacrine in lithium chloride (LiCl)-pretreated animals (Bagetta et al., 1992, Eur. J. Pharmacol., 213: 301–304), thus suggesting that abnormal accumulation of NO in the brain may lead to epileptogenic and neurodegenerative disorders. We now report further experimental evidence which supports this hypothesis. In rats pretreated (24 h before) with LiCl (12 mEq/kq i.p.) tacrine (5 mg/kg i.p.) administration produced an approximately 70% increase in hippocampal NO synthase activity 15 min after treatment. A 3.5 fold raise in hippocampal cGMP levels was also observed. Atropinc (5 mg/kg i.p. given 15 min before tacrine) prevented the increase in NO synthase activity produced by tacrine and LiCl. Prevention of the NO synthase increase was also shown following the microinjection of L-NAME (300 μg) into one lateral cerebral ventricle 10 min before tacrine administration. Administration of tacrine (5 mg/kg i.p.) alone did not significantly affect hippocampal NO synthase activity; LiCl (12 mEq/kg i.p.) increased the enzyme activity by approximately 35% in the hippocampus of rats receiving 24 h later the vehicle (1 ml/kg i.p.) used to dissolve tacrine. Both basal NO synthase activity and that increased by LiCl-tacrine were significantly reduced when influoroperazine (100 μM), an inhibitor of calmodulin-mediated functions, was present in the hippocampal homogenate. Similarly, addition of EGTA (1 mM) reduced (by approximately 80%) NO synthase activity; however, no futher reduction was obtained when L-NAME (1 mM) was also added, thus indicating the Ca^{2+}-calmodulin-dependent nature of hippocampal NO synthase.

Nutrition

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Determination of the true ileal digestibility of pea amino acids by means of ^{15}N-labelled diets or animals

Pigs (2 × 4) fitted with an ileal PVTC cannula were fed, twice a day (8–20 h), with a diet composed of peas (60%), starch, sucrose, minerals and Cr_2O_3. Four received a blood perfusion of an ^{15}N-leucine (95% enrichment) solution (5 mg/kg $W^{0.75}$ · day) for 10 days. The ileal digesta were collected in the last 3 days. The endogenous N content of the digesta collected from 4 to 8 h after the meal, was determined by measuring the ^{15}N-dilution in the digesta, compared with the ^{15}N-enrichment of the blood TCA-soluble fraction. The digesta Cr and N contents were used to calculate the endogenous and dietary N flows. The amino acid (AA) profile of the endogenous fraction was estimated by means of an N-free diet and was used for the calculation of the true AA digestibility.

The other pigs received one single ^{15}N-labelled meal (0.95% enrichment), the following meals being unlabelled. The digesta were collected for 3 days in a row, including the one with labelled meal. Each collected sample was analysed for ^{15}N in order to regroup those with the highest enrichments (= samples collected from 4 to 8 h after the meal, with the highest dietary AA contents). The AA of these samples were separated by preparative IEC, purified on an Amberlite column and analysed for ^{15}N with an elemental N analyser (Roboprep) coupled to an IR-MS (Sira 12, VG). On basis of the Cr, ^{15}N and AA contents, the endogenous/dietary N and AA flows and the apparent/true ileal digestibilities of the pea AA were then calculated.

The apparent N digestibilities were similar for both methods (72.5% for ^{15}N perfusion vs 72.6%), whereas the endogenous N content of the digesta was significantly higher for the ^{15}N-perfusion technique (71.7% total N vs 54.6%). Therefore, the true ileal N digestibility was also higher for this method (92.9 vs

86.1%). With labelled diets, the values reach those obtained by the classic method (apparent digestibility + N-free diet). In this case, a contamination of the endogenous compounds by ^{15}N-AA is possible and can induce a slight underestimation of the true digestibilities.

Both methods still need a validation but from the literature, the ^{15}N-perfusion technique seems to overestimate the endogenous N content of the digesta and thereby, the true digestibilities.

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Amino acid composition, protein quantity and protein quality of finnish foods

This lecture is about a composition study carried out to determine amino acid composition of the most important foods produced in or imported to Finland. The amino acids of 150 food items were determined by ion-exchange chromatography (LKB 4150 Alpha) or by fluorometer (tryptophan) after acid or alkaline (tryptophan) hydrolysis. The protein contents of samples were determined by the Kjeldahl nitrogen determined method. The purpose of this study was also to calculate new protein values: protein content as a sum of amino acid residues and a relative protein value.

The new protein values (the sum of amino acid residues) are smaller than the protein values determined by the Kjeldahl method (crude protein values) because of nonprotein compounds, which are included in crude protein. The new protein value of milk and milk products were 2–28% smaller than the crude protein. The average difference between these two protein values was approx. 8%. The difference was greatest in human milk sample and smallest among fermented liquid milk products and most cheeses. In meat and edible offals the differences between new and crude protein values were greater than in milk and milk products. The differences varied from 2 to 28% and were on the average approx. 15%. The smallest difference was in the steer blood sample and greatest difference in the chicken sample. In meat products the differences were smaller varying from 2 to 18% (on the average 8%). Also in fish and in fish products the differences between protein contents were great (9 to 29%). On the average it was 20%. In cereal samples, wheat, barley, rye and oat the differences between new and crude protein values were small, 0.4–1.3% (the exception was wheat bran, 27%). In vegetables and fruits the differences varied considerably from 4 to 45% and were on the average approx. 23%. The greatest differences occurred in carrot, salted trivial milk-cup (*Lactarius trivialis*) and lettuce samples 35, 34 and 45% respectively. In tomato and corn the differences were small 0 and 4%. In fruits the differences were more similar to each other and varied between 12 to 25%.

The relative protein value was also calculated from the amino acid contents determined. That is the ratio of the amount of essential amino acids plus arginine to the amount of the non-essential amino acids plus cysteine and tyrosine. The relative protein value should be near 1 to obtain optimum growth responses in rats. In food proteins the relative values varied a lot. In milk and in fermented liquid milk products the value was 0.8–0.89 and in cheeses 0.79–0.94. The value of human milk was 0.89. In meat, edible offals and meat products the relative protein values were 0.87–1.06, 0.88–1.19 and 0.76–1.11 respectively. In fish and in fish products these values varied from 0.78 to 1.07.

Because the total essential amino acid content was relatively low in cereals, most vegetables and fruits the relative protein value was quite low. The value varied from 0.50–0.75 in all cereals other than rice which was 0.86. In vegetables the values were also quite low 0.37–0.56. Exceptions were potato 0.72, lettuce 0.80, onion 0.82, green bean 0.75, spinach 0.94, pea 1.07 and salted trivial milk cup (*Lactarius trivialis*) 1.02. The relative protein value does not fully indicate the nutritional value of protein. Also limiting amino acids must be considered.

The use of new protein values in calculations of good energy values, methods for calculating limiting amino acids and evaluating the nutritional value of proteins are also discussed.

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Extraction, solubility, DISC-electrophoretic and nutritional properties of proteins isolated from "cachaz" seeds (*Okenia hypogaea* Schle. and Cham)

"Cachaz" seeds (*Okenia hypogaea*) can represent a good source of protein (14–16%) to human consumption. Information on the extraction of proteins could not be found in the literature. In this study the proteins were extracted by a modified Osborne's procedure and the solubility, DISC-electrophoretic and some *in-vitro* nutritional properties were evaluated.

The results showed that the cachaz proteins could be isolated and fractionated with Ac-COOH/KOH buffer (I = 0.01), PO_4/NaCl (1.0 M)/DL-DDT, Isopropanol (55% v/v) and NaDodSO$_4$/NAOH (0.01 M) as solvents in the modified procedure.

The extraction method developed for cachaz proteins showed the importance of factors such as temperature, time and number of extractions on the total protein recovery; these value was 97%. The main fraction were glutelins (43.3%) follow by globulins (28.2%) and albumins (20.8%) with molecular weights between 10–15 Kd to albumins (4 fractions); 90–100 Kd to globulins (6 fractions) and 110–200 Kd to glutelins (4 fractions); the DISC-electrophoretic pattern of globulins were similar to 7s and 11s fractions of *dicotiledonae* seeds.

Finally, aminoacid profile was similar to FAO/WHO Standar with acceptable levels of lysine, tryptophan and sulphur aminoacids wich are found in low proportions in legume and cereal grains; Chemical Score, *in vitro* digestibility and C-PER values were hight related with other seeds.

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Competitive adsorption to air-water interface of peptides obtained by tryptic hydrolysis of β-lactoglobulin

In previous work, two peptidic fractions (F1 and F3) were isolated from a tryptic hydrolysate of β-lactoglobulin (β-lg) by anionic exchange chromatography. These fractions were further identified as responsible of improved interfacial properties of the hydrolysate. The fraction F3 was composed of 95% peptide β-lg 41–60 and the fraction F1 was made up of 50% peptides β-lg 21–40 and β-lg 25–40. These peptides are characterized by a distribution of hydrophobicity in discrete regions (three to five residues) separated by polar residues (two or three).

Using Wilhelmy plate method, rates of adsorption (mNm^{-1} s^{-1}) of β-lactoglobulin, α-lactalbumin (α-la) and both peptidic fractions were measured (pH 4 or 7, $\mu = 0$ or 0.6) separatly or in combination in order to evaluate the competitive adsorption of these protein components at an air-water interface.

The rates of adsorption of β-lg and α-la were generally higher at acidic pH and improved by increasing ionic strength of the protein solution. The same behaviour was observed for peptidic fractions. At pH 4, adsorption rates of β-lg-peptides (F1 and F3) were higher than native β-lg corresponding to an increase over β-lg adsorption rates by a factors ranging from 2 to 11. Most of protein-protein or protein-peptide combinations had little effect on adsorption rates of each component. However, competitive adsorption was observed for the F1 + F3 mixture. At pH 4 $\mu = 0$, F3 adsorbed preferentially at the interface while an increase of ionic strength ($\mu = 0.6$) favoured the adsorption of F1. The results are discussed in terms of the impact of hydrophobicity and net charge of the peptides on their interfacial properties.

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On the use of membrane technology for the separation of peptides and amino acids from milk protein hydrolysates

Milk proteins are distributed in two major groups, namely the caseins fractions (α_{S1}, α_{S2}, β, κ and γ) which represent 80% of the total protein content, and whey proteins (β-lactoglobulin, α-lactalbumin, serum albumin and immunoglobulin) which represent the remaining 20%. The enzymatic hydrolysis of caseins and of whey proteins is industrially practiced for the preparation of products designed for post-surgery clinical nutrition, and for hypoallergenic infant formulas. Also, specific peptidic sequences of milk proteins have been recently recognized as having biological properties such as opoïd, hypotensive or mineral carrier. This latter breakthrough, while opening the field of milk protein hydrolysates to pharmacology or biomedecine, raises the technological challenge of producing biologically active peptides.

Our previous work showed that a 30 KD polysulphone ultrafiltration membranes can successfully achieve the removal of the enzyme and non-hydrolysed protein from a reaction mixture, while a 1 KD membrane can fractionate the mixture of peptides into a concentrate of medium range (2000 daltons > MW < 5000 daltons) molecular weigth and a permeate of low range (> 2000 daltons) peptidic fractions. Our most recent work shows that polysulfone ultrafiltration membrane do not only achieve the separation of peptides via molecule size sieving effect, but it also possesses surface properties that impairs the passage of charged peptides or amino acids. In fact, the very rapid flux decline during ultrafiltration reflects the possible occurence of membrane-peptides interactions. The results are discussed in the scope of their impact on the potential of membranes for the separation of peptides and are compared to those obtained by existing chromatographic methods.

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Plasma amino acids in ESRD patients on hemodialysis and milk-hydrolysate supplemented diet

Insufficiency of suitable and well balanced protein foods for ESRD patients on hemodialysis is a problem, which we commonly face. As an attempt to solve it partially we apply a newly developed protein concentrate on a milk base, with a protein content of approximately 60%. The quality assessments include: the amino acid composition of protein, the essential/total amino acids ratio, the amino acid score, the Lys/Arg ratio, as well as the plasma amino acid pool and the Trp/LNAA and Val/Gly ratios.

Twenty ESRD patients (12 F and 8 M) without intercurrent illnesses were treated by a 4-hour acetate hemodialysis thrice weekly. The patients were supplemented with 0,2–0,5 g protein concentrate/kg body mass daily for a period of 3 months. We can point as a positive result the final elevation of Ile, Leu, Lys and Tyr in comparison with their starting levels. Val keeps its level, which shows that its quantity in the milk hydrolysate is sufficient to cope with the losses.

It could be assumed that with the proposed protein supplementation in chronic renal impairment it is possible to correct the changes in plasma concentration of some AA.

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Tryptophan and tyrosine in stress exposed humans – nutritional implications

Experimental studies provide evidence for nutritional modulations of brain neurotransmitter synthesis by dietary intake of essential amino acids tryptophan and tyrosine (phenylalanine). Plasma essential and nonessential amino acids levels in stress exposed subjects were investigated. Plasma ratios of tryptophan to the sum of large neutral amino acids (LNAA) – Val, Leu, Ile, Met, Phe and Tyr were determined. Statistical differences in Trp/LNAA of stress exposed subjects, 0,076 ± 0,036 (mean ± 2 SD) versus controls, 0,106 ± 0,082 were found ($p < 0,05$). Nutritional control of brain neurotransmitter levels, underlying specific behaviours, suggests the possibility of dietary implication to provide behavioural modulations. Assessment of tryptophan and tyrosine content of common Bulgarian protein – rich foods was carried out. Nutritional considerations include protein versus carbohydrate meals and circadian – rhythms temporal distribution pattern.

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Amino acids as ligands of cations in food chemistry

The presence of metallic cations in food is frequently considered dangerous under the nutritional point of view, but in many cases it can play even a therapeutical role.

Positive or negative effects on the human health of the presence of metal in food can depend on the type of metal, its total quantity, predomining chemical species under which it is present in food.

The aim of our research points to find out in which form cations like copper(II), lead(II), cadmium(II) and zinc(II) can be present in aqueous solutions containing different kinds of aminoacids.

Glycine, α- and β-alanine, serine, aspartic and glutamic acids, histidine, ornithine, cysteine and triptophane as ligands of the above cations have been studied in 1.00 M NaCl and/or 1.00 M $NaClO_4$.

In aqueous solutions (in constant ionic medium), the free concentration of copper(II), lead(II), cadmium(II) and zinc(II) was experimental measured by means of suitable amalgam elec-

trodes and on the same solutions, the free hydrogen ion concentration was also measured by using a glass electrode.

Experimental data could be explained by assuming the presence of mononuclear complexes in the cation, of the type ML_n. However complexes with participation of protons were also formed. It seems remarkable that even in neutral or moderately alkaline (ph ~8–9) solutions, protonated complexes are still present.

The behaviour of zinc(II) was a subject of specific investigation.

The inspection of polarographic analysis of aqueous solutions equilibrated with homogenized flour or grain shows also the presence of zinc(II). However the wave relative to this cation appears at more negative $E_{(1/2)}$ than the corresponding on measured in 1.00 M NaCl.

To find an explanation of the shift of $E_{(1/2)}$, several series of polarographic analysis of solutions containing zinc(II) and different aminoacids were carried out and the results were compared with those obtained from solutions equilibrated with flour or grain.

Among the studied aminoacids, histidine seems to give shifts of $E_{(1/2)}$ comparable with those obtained for grain.

This study will be a subject of further investigation.

Erratum

The following abstract was published in Amino Acids (1993) 5/1: 116 without authors' names

Synthesis and Biosynthesis

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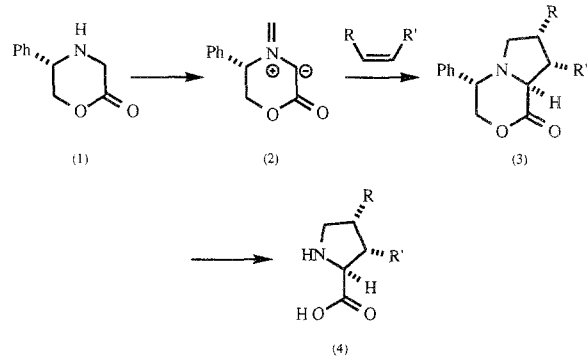
Synthetic approaches to functionalised pyrrolidines

This research concerns the application of a [3 + 2] cycloaddition reaction to the synthesis of chiral functionalised pyrrolidines, and attempts to elaborate these to a class of organic compounds known as the kainic acid family. Kainic acid and closely related congeners – domoic acid and acromelic acid – have attracted considerable interest due to their potent insecticidal, anthelmintic and neuroexcitatory properties. The morpholinone (1) reacted with paraformaldehyde to give the chiral, stabilized azomethine ylid (2) which was trapped by a dipolarophile to give cycloadducts of type (3) (see scheme below).

The morpholinone (1) was reacted in this manner with a range of dipolarophiles. NOE difference experiments have permitted the assignment of the *endo*- and *exo*-stereochemistries of the adducts obtained. In addition, three structures have been confirmed by x-ray crystallographic analysis.

Electron-poor alkenes (eg where R is a carbonyl functionality) gave higher yields than electron rich alkenes (eg where R is a phenyl group). In all the reactions discussed there was complete facial selectivity with respect to the azomethine ylid; approach of the dipolarophile was always from the face opposite to the bulky phenyl group of the ylid. The observed regioselectivities were, in general, what was expected from application of FMO theory. The dominant stereochemistry of addition was *exo*.

A number of these cycloadducts were then deprotected by hydrogenolysis to give the free amino acids (4). The elaboration of these products towards kainic acid, and related structures, is currently under review.



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