Biosynthesis of deuteriated amino acids by *Candida lipolytica*[1]

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Summary. Biosynthesis of a whole series of deuteriated amino acids has been carried out, by cultivation of the yeast *Candida lipolytica* in an artificial medium composed of a deuterio-alkane, heavy water, water and some mineral salts.

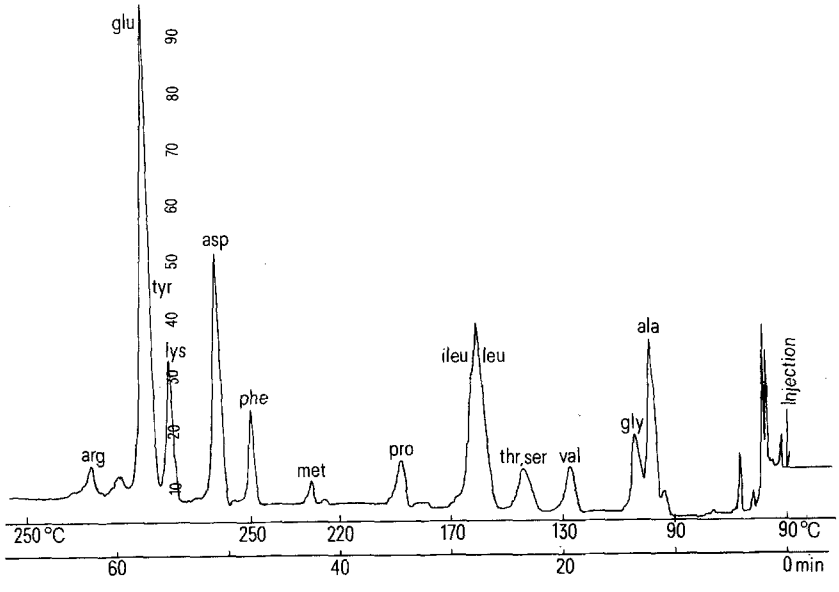
The biological effects of deuterium on microorganisms[3-6] constitute a part of the research work in our laboratories. The need for well-defined isotope-labelled compounds in the studies of biological systems, such as cell membranes, requires a continuous development of convenient methods for synthesizing and biosynthesizing these substances.

Synthesis of deuteriated amino acids has been attempted by us, via protium-deuterium exchange occurring between sodium amino-carboxylates and deuterium oxide (heavy water) in the presence of sodium deuterioxide, deuterium-reduced Adams platinum oxide and alumina. However, some elementary reactions involved in this catalytic exchange result in racemisation of the end products. Thus, racemic deuteriated amino acids were obtained, which were not so useful for biological studies.

It is known that there is a wide variety of microorganisms which can grow on hydrocarbons, specifically on alkanes, to produce food proteins[7]. Among the yeasts, *Candida lipolytica* is known to utilise a wide range of normal-chain alkanes as sole carbon source. As our laboratories had already synthesized a homologous series of saturated deuterio-n-alkanes - via H_2-D_2 catalytic exchange in an autoclave[8-12] or via Kolbe anodic condensation of deuteriated carboxylic acids[13,14] - it was convenient for us to use these deuteriocarbons and *C. lipolytica* for the attempted biosynthesis of deuteriated proteins. In an early attempt the yeast was grown on an emulsion composed of perdeuterio-n-heptadecane (over 98.5% deuterium content[10]) and a synthetic medium[15], which consisted of soluble mineral salts and water. The fungus growth was performed using a previously described fermentation vessel[16] equipped with a stirrer working at 800 rpm. During the growth period of 30 days the culture medium was kept at a constant temperature of 30 °C, with stirring and with pure-air bubbling. Since the amino acids derived from the biosynthesized proteins possessed a low deuterium content, a protium-deuterium exchange seemed likely to have been involved during the biosynthetic reactions. On the other hand, it has been proved that H_2-D_2 exchange does not occur between heavy water and n-heptadecane at 240 °C, even in the presence of favourable catalysts such as deuterium-reduced platinum oxide and sodium deuterioxide[11].

In order to increase the deuterium content of the biosynthesized proteins the fungus was trained to subsist in media containing heavy water as described below. Here we give an example of *C. lipolytica* culture which was carried out in the artificial medium composed of perdeuteriated-n-eicosane (over 98% D content, m.p. 34.2 °C), salt mixture (7 g KH_2PO_4, 0.2 g $MgSO_4$, 5 g NH_4Cl and 0.1 NaCl per liter) and 50:50 v/v water-heavy water (99.8% D_2O), at 36-37 °C under the conditions cited above. When the growth was accomplished, the lipids formed and the unconsumed deuterio-alkane were extracted with light petroleum. The culture medium was then saturated with NaCl, and the yeast together with precipitate was removed by paper filtration. After drying over silicagel in a desiccator the protein mass was submitted to hydrolysis with HCl 6 N at 110 °C during 18 h. A small part of the amino acid mixture recovered from this procedure was subjected to Moore-Stein analysis. The qualitative and quantitative composition of the amino acid mixture is shown in the table. The amino acids from another part of the mixture were converted into (N(O)-heptafluorobutyryl-O-isoamyl derivatives[17] and analysed by combined gas-liquid chromatography and mass spectrometry (GC/MS). The gas-liquid chromatogram is shown in the figure, which gives a qualitative picture. Isotopic enrichment of some amino acids was calculated from the mass spectrum of the corresponding heptafluorobutyryl-isoamyl derivative. These compounds had an average deuterium content of 51%.

For the purpose of creating deuteriated yeast strains for



Gas-liquid chromatogram of a mixture of N(O)-heptafluorobutyryl-O-isoamyl amino-carboxylates, from which the corresponding amino acids were derived from *Candida lipolytica* grown on deuteriated substrate. This qualitative analysis was performed with a LKB-9000 gas chromatograph-mass spectrometer. Conditions: glass column of 4 m length and 3 mm inside diameter filled with 1% SE-30 on 100-120 mesh Chromosorb W, helium flow 20 ml/min, ionizing energy 70 eV, trap current 60 μA, molecular separator at 260 °C, and ion source at 260 °C.

Result of the Moore-Stein analysis of amino acid mixture deriving from *Candida lipolytica* grown on deuteriated substrate

Lysine	31.32 nmoles/ml
Histidine	11.06 nmoles/ml
Arginine	20.65 nmoles/ml
Tryptophan*	
Aspartic acid	55.55 nmoles/ml
Threonine	47.17 nmoles/ml
Serine	44.25 nmoles/ml
Glutamic acid	53.73 nmoles/ml
Proline	12.85 nmoles/ml
Glycine	54.49 nmoles/ml
Glucosamine	60.00 nmoles/ml
Alanine	61.74 nmoles/ml
Valine	32.50 nmoles/ml
Cystine**	4.04 nmoles/ml
Galactosamine	15.00 nmoles/ml
Methionine**	6.64 nmoles/ml
Isoleucine	14.44 nmoles/ml
Leucine	41.41 nmoles/ml
Tyrosine	15.34 nmoles/ml
Phenylalanine	21.40 nmoles/ml

* Compound destroyed during hydrolysis. ** Compound partly destroyed during hydrolysis.

culture in media containing a high concentration of heavy water, *C. lipolytica* was grown and transplanted successively on gelatinous media in Petri dishes. These media were composed of a normal long-chain deuterio-alkane, the above mentioned mineral salts, vitamins[18], agar-agar, and mixtures of water-heavy water of the following proportions (by v/v): 50:50, 40:60, 35:65, 30:70, 27.5:72.5 and 20:80. In these cultures the fungus-cell size was, on average, larger than that originating from a non-deuteriated culture in the same growth period. This phenomenon became more apparent when the deuterium content of the water in the culture media reached the lethal levels for the yeast – beyond 80% D_2O. However, frequent subculturing on substrates with increasing D_2O concentration may result in a higher adaptability of the fungus toward deuterium, thus the expected yield of deuteriated amino acids could be increased. Further development of the biosynthesis along these lines is now in progress, and the results will be communicated later.

The present results indicate that *C. lipolytica* can assimilate the perdeuteriated alkanes as its sole carbon source for biosynthesis. This fact is important, not only because it leads to a suitable approach for obtaining deuteriated lipids and amino acids by a biosynthetic method, but also because it offers new possibilities for tracing microbial metabolic pathways by the use of isotope-labelled substrates with subsequent analysis by GC/MS.

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Correlations between glucose-inhibition and control parameters of α-glucosidase kinetics in *Apis mellifica* haemolymph (Hymenoptera: Insecta)

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Summary. Kinetics studies of haemolymph α-glucosidase inhibition by D. glucose led to general correlations between inhibition and control parameters, all over the honeybee development. Maximum velocities are not affected by the inhibition, while affinity constants are always significantly increased, and Hill coefficients tend to decrease, especially in foraging adults and in prenymphs. In this later case, the 'n-type' effect tends to break the manifestation of the 'K-type' mechanism.

The haemolymph α-glucosidase contributes to the maintenance of Insects glycaemic levels, and we have shown that D. glucose is a natural inhibitor of this enzyme in honeybees haemolymph[1]. Evidence was given for a 'K-type' (competitive) inhibition in emerging adults[2] and for a mixed mechanism rather affecting the Hill coefficient in young nymphs[3]. Other cases of glucose inhibition have been reported by Sinha[4] for midgut invertase and by Giebel and Domnas[5] for whole larvae trehalase, both in Diptera, but the kinetics mechanisms have not been studied. Previously to mathematical studies of the 'n-type' molecular transitions, it seemed necessary to determine more thoroughly the