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The Irreversibility of the Deamination of Threonine in the Rabbit and Rat

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It has been known for some time from perfusion and growth experiments with mammals that many amino-acids are deaminated to keto acids and that these can be reconverted to the original amino-acids. Thus, the fact that many essential amino-acids can be replaced in the diet by the D-enantiomorphs or by the corresponding hydroxy or keto acids indicates that deamination and reamination occur readily. However, the extent and the speed of such reactions was not fully realized until studies with isotopic nitrogen were initiated in the field of intermediary metabolism (for reviews see Schoenheimer, 1942; Schoenheimer & Rittenberg, 1940). It was shown that, after feeding to rats ammonium salts, glycine, L- or D-leucine or L-tyrosine labelled with ^{15}N , almost all the amino-acids isolated from the proteins of different tissues contained some excess ^{15}N . The amide nitrogen and the amino nitrogen of glutamic and aspartic acids always had the largest concentrations of the isotope, but some 'essential' amino-acids such as histidine and leucine also exchanged reversibly their α -amino nitrogen with that of other amino-acids or with ammonia. For other essential amino-acids such as tryptophan, methionine and phenylalanine, such a reversible deamination and reamination has not been demonstrated by the isotope method, but positive evidence for the occurrence of such reactions has been obtained from growth experiments using D-amino-acids and also related keto and hydroxy acids (for reviews, see Rose, 1938; Jackson & Chandler, 1939; Neuberger, 1948). Negative evidence from such nutritional experiments is not conclusive. Thus D-valine, D-leucine and D-isoleucine are not available for growth in the

rat (Rose, 1938), and similar results have been reported for the mouse (Bauer & Berg, 1943). The chick can utilize D-leucine, but not D-isoleucine or D-valine (Grau & Peterson, 1946). On the other hand, Schoenheimer, Ratner & Rittenberg (1939), using L-leucine labelled with ^{15}N and deuterium, showed that this amino-acid exchanges its nitrogen with that of other amino-acids. Later, the inversion of D- to L-leucine in the rat was clearly demonstrated by the method of double labelling (Ratner, Schoenheimer & Rittenberg, 1940). These findings and the observation of Rose (1938) that the three keto acids corresponding to leucine, valine and isoleucine support growth indicate that these three essential aliphatic amino-acids yield nitrogen to, and accept nitrogen from, other amino-acids. The negative results of growth experiments with the D-amino-acids may be explained by assuming that the rate of deamination is too slow for the requirements of growth.

The only amino-acid which had been isolated by the Columbia workers and which never contained any excess ^{15}N was lysine (Foster, Schoenheimer & Rittenberg, 1939). It had been shown earlier (Foster, Rittenberg & Schoenheimer, 1938) that lysine, unlike other amino-acids, does not take up deuterium from the body water. By the method of double labelling (deuterium and ^{15}N) it was demonstrated that the products of deamination of lysine cannot be aminated again to form lysine (Weissman & Schoenheimer, 1941). This conclusion agrees fully with the observations that neither D-lysine (Berg, 1936) nor any known derivative of lysine, with the exception of ϵ -N-methyllysine and ϵ -N-acetyllysine

(Neuberger & Sanger, 1943, 1944), can replace L-lysine in the diet.

No evidence from isotope experiments was available to indicate whether the one remaining essential amino-acid, threonine, is reversibly deaminated in the mammalian organism. The finding of West & Carter (1938) that D-threonine is not available for growth in the rat could be taken to indicate that threonine resembles lysine in this respect. But as argued above, a rigorous conclusion cannot be drawn from such evidence. One of us (Elliott, 1949) has recently described a method which permits the isolation of threonine from relatively small quantities of protein. It was therefore decided to investigate whether threonine accepts nitrogen from other amino-acids. The first experiment was done with a rabbit, using a relatively small quantity of ^{15}N -containing glycine; the second experiment was carried out with a group of rats, using a relatively large amount of labelled amino-acid.

A preliminary communication on this work has been made by Elliott & Neuberger (1949).

EXPERIMENTAL

Rabbit experiment. Two male Hollingsworth half-lopped rabbits (1.61 and 1.65 kg. body wt.), which were being fed the Institute stock diet, were given glycine containing 31.69 atom percentage excess ^{15}N mixed with the food in divided doses over 4 days. The total amount of labelled glycine given was 0.4 g./kg. body wt. The animals were killed on the fifteenth day after the last administration of the isotopic compound. The muscles of the two animals were removed and minced. The protein was separated by the salting-out method of Deutsch, Eggleton & Eggleton (1938). The resulting mixed muscle protein was heated with several lots of water until the aqueous solution was free of SO_4^{--} and was then treated successively with warm ethanol and ether.

A portion (50 g.) of the air-dry protein was then hydrolysed and serine, threonine and glutamic acid were isolated as described by Elliott (1949); 1.34 g. of a mixture consisting of 56% threonine and 44% serine was obtained. This was recrystallized from 80% (v/v) aqueous ethanol and 120 mg. of the material was fractionated on a starch column giving 65 mg. of L-threonine and 47 mg. of DL-serine. Both samples were recrystallized for analysis. The threonine gave a titration figure of 99.4% of the theoretical value (average of three analyses) by the periodate method (Rees, 1946), whilst the serine gave 96.4% (average of three analyses). The glutamic acid hydrochloride was recrystallized from aqueous HCl and the free amino-acid was isolated by exact neutralization of the hydrochloride and recrystallized from water-ethanol. Glutamic acid had m.p. 201° (uncorr.); $[\alpha]_D^{20} + 31.5^\circ$ (c, 2.0 in 5.5N-HCl). (Found: N, 9.4. Calc. for $\text{C}_5\text{H}_9\text{O}_4\text{N}$: N, 9.5%.) Tyrosine was obtained from the neutral amino-acid fraction and recrystallized three times from water. It had $[\alpha]_D^{21} - 7.9^\circ$ (c, 1.5 in 5.5N-HCl). (Found: N, 7.5. Calc. for $\text{C}_9\text{H}_{11}\text{O}_3\text{N}$: N, 7.7%.)

Another portion of the air-dry protein (50 g.) was hydrolysed by refluxing with 300 ml. of 5.5N-HCl for 12 hr. Excess HCl was removed, the basic amino-acids were precipitated with phosphotungstic acid and most of the glutamic acid was

obtained as the hydrochloride. The acidic solution was concentrated to dryness and the glycine was obtained as the trioxalatochromiate complex (Bergmann & Niemann, 1938). The complex was decomposed and the glycine benzoylated. The hippuric acid (1.32 g.) was crystallized and recrystallized from water; it had m.p. (uncorr.) 189° . (Found: N, 7.7; Calc. for $\text{C}_9\text{H}_9\text{O}_3\text{N}$: N, 7.8%.)

Rat experiment. One male and two female rats of body wt. 250, 235 and 215 g., respectively, were kept on the following diet which was fed *ad lib.* for 18 days before isotopic glycine was given: salt mixture (U.S. Pharmacopoeia 1947, p. 721, no. 2), 4%; yeast powder, 5%; casein, 3%; maize starch, 80%; arachis oil, 6.4% and cod liver oil, 1.6%. The protein content of the diet was about 5.5%. At the end of the preliminary period the rats had lost 10, 3 and 12 g. respectively. The rats then received on three successive days 150 mg. of isotopic glycine/rat/day mixed with the food. The glycine had 31.5 atom percentage excess ^{15}N . The rats were killed 24 hr. after the last administration of the labelled glycine. Liver, spleen, kidney, pancreas, heart and the washed small intestine of the three rats were combined and minced. The product was heated with 50% (v/v) ethanol and the resulting precipitate was repeatedly washed with the same solvent. Fat was removed by successive treatment with ethanol, acetone and ether. 10.45 g. of air-dry material was obtained.

The protein was hydrolysed and the basic amino-acids were removed with phosphotungstic acid. Glutamic acid was isolated as the hydrochloride; this fraction was unfortunately lost. Tyrosine was obtained from the neutral amino-acid fraction; the recrystallized material had $[\alpha]_D^{18} - 7.8^\circ$ (c, 1.5 in 5.5N-HCl). (Found: N, 7.6. Calc. for $\text{C}_9\text{H}_{11}\text{O}_3\text{N}$: N, 7.7%.) From the basic fraction arginine was obtained as monofluoroborate; this was converted with $\text{Ba}(\text{OH})_2$ to the monohydrochloride, $[\alpha]_D^{20} + 26.1^\circ$ (in terms of free amino-acid) (c, 1.4 in 5.5N-HCl). (Found: N, 26.4. Calc. for $\text{C}_6\text{H}_{15}\text{O}_2\text{N}_4\text{Cl}$: N, 26.6%.) Histidine was removed from the mother liquor obtained in the precipitation of arginine by Ag^+ ions and the lysine reprecipitated as phosphotungstate after removal of NH_3 by aeration. The lysine phosphotungstate was converted to the picrate which was recrystallized, m.p. (decomp.) 265° . The picrate was decomposed with HCl and the lysine crystallized as monohydrochloride which was recrystallized from aqueous ethanol, $[\alpha]_D^{20} + 25.5^\circ$ (c, 1.32 in 5.5N-HCl) (in terms of the free amino-acid). (Found: N, 7.6. Calc. for $\text{C}_6\text{H}_{13}\text{O}_2\text{N}_2\text{Cl}$: N, 7.6%.)

The serine threonine mixture, which was obtained as described by Elliott (1949) and amounted to 170 mg., was recrystallized. By periodate titration (Rees, 1946) it was found to contain 73.7% threonine and 24.5% serine. The recrystallized material (100 mg.) was placed on the starch column. Yield of threonine was 46 mg., which was recrystallized for analysis. This product titrated to 100% of the theoretical value by the periodate method (average of three analyses). The column, after most of the threonine had passed through, blocked up and serine could not be isolated. Isotope analyses were therefore done on the serine threonine mixture and the values of serine were calculated after allowing for the threonine present.

In this experiment glycine was obtained from that fraction of the hydrolysate which originally contained oxazolines derived from serine and threonine, and benzimino-ethyl ether derivatives mainly of glycine, alanine and valine. As shown by Elliott (1949), alkali converts these latter into the corresponding benzamido acids, whilst the two oxazolines

are stable to the alkali treatment used, but are converted by the subsequent acidification to *O*-benzoyl amino-acids which, unlike the *N*-benzoyl compounds, are not extracted into organic solvents from the acidified aqueous solution. The mixture of *N*-benzoyl compounds from the first ethyl acetate extract was hydrolysed by refluxing with 5.5N-HCl for 8 hr. Benzoic acid was removed by filtration and extraction with ether. The aqueous solution was concentrated to dryness, and the residue taken up in water (10 ml.). K oxalatochromiate (4.5 g.), ethanol (17.5 ml.) and 10% (w/v) HCl (2.5 ml.) were then added. The mixture was shaken for 6 hr., filtered, and the precipitate washed with 12.5 ml. of 75% (v/v) ethanol. The precipitate was dissolved in water (9 ml.) to which 1.3N-NaOH (7.5 ml.) had been added. Benzoylation was carried out in the usual manner using 1.87 ml. of benzoyl chloride and 10 ml. of 1.3N-NaOH. After acidification excess benzoic acid was extracted with benzene and hippuric acid with ethyl acetate. The ethyl acetate solution was dried and concentrated to dryness. The residue was crystallized and recrystallized from water. The hippuric acid had m.p. 190° (uncorr.). (Found: N, 7.6. Calc. for C₉H₉O₂N: N, 7.8%.)

RESULTS

Rabbit experiment (Table 1). In this experiment the amount of isotopic glycine fed was relatively small. It is not surprising, therefore, that the concentration of ¹⁵N even in glycine was rather low.

Table 1. *Atom percentage excess ¹⁵N of amino-acids isolated from the muscle proteins of two rabbits fed isotopic glycine*

Amino-acid	Atom percentage excess ¹⁵ N
Glycine	0.092
Serine	0.086
Glutamic acid	0.024
Threonine	0.000
Tyrosine	0.014

Serine had almost the same value for atom percentage excess ¹⁵N as glycine. The corresponding figures for glutamic acid and tyrosine were considerably lower, but well outside the limits of error of the mass spectrometric analysis which are about ± 0.005%. Threonine contained no excess ¹⁵N.

Rat experiment (Table 2). In this experiment the amount of isotopic glycine administered/kg. body weight was about four times larger than in the rabbit

Table 2. *Atom percentage excess ¹⁵N of amino-acids isolated from the proteins of the internal organs of three rats fed isotopic glycine*

Amino-acid	Atom percentage excess ¹⁵ N
Glycine	3.500
Serine	3.300
Threonine	0.000
Arginine	0.366
Tyrosine	0.107
Lysine	0.000

experiment. Moreover, it was to be expected, on the basis of the results of Shemin & Rittenberg (1944),

that the concentration of the isotope in the protein of the internal organs immediately after the administration of the labelled compound would be much higher than the corresponding values for muscle more than 2 weeks after the beginning of the experiment. The ¹⁵N values were, in fact, about 10–30 times higher than in the first experiment. Glycine and serine contained very high, and again very similar, amounts of ¹⁵N. Both arginine and tyrosine appeared to have exchanged their nitrogen with that of glycine, whilst lysine and threonine showed no excess of ¹⁵N.

DISCUSSION

The results show clearly that, in the rat, threonine isolated from tissue proteins after feeding ¹⁵N-labelled glycine does not contain any excess ¹⁵N, whilst all other amino-acids isolated, except lysine, contain relatively large amounts of the labelled nitrogen. If a nitrogen-free derivative of threonine can be reaminated at all to form threonine, the speed of the reaction must be exceedingly slow, but this possibility cannot be completely excluded. It appears more probable that deamination of threonine, like that of lysine, is irreversible in the rat and that either threonine is not converted into the corresponding α-keto acid or the keto acid is not reaminated. The fact that the L-amino-acid oxidases of rat kidney and liver do not attack L-threonine (Blanchard, Green, Nocito & Ratner, 1944) may indicate that α-keto-β-hydroxybutyric acid is not a metabolite of threonine in the rat. With the rabbit the evidence is less conclusive, since the amount of isotopic glycine fed was smaller; but the data suggest that in the rabbit too, deamination of threonine is not reversible. It thus appears that threonine and lysine differ from other essential amino-acids, which, like the non-essential amino-acids, take part in a reversible exchange of α-nitrogen.

Another interesting point emerges from this work. Shemin (1946) has conclusively demonstrated that serine is converted to glycine in the rat and guinea pig. The converse reaction, transformation of glycine to serine, has also been shown to occur in whole rats (Sakami, 1948; Goldsworthy, Winnick & Greenberg, 1949), rat-liver slices (Siekevitz, Winnick & Greenberg, 1949) and rat-liver homogenates (Winnick, Moring-Claesson & Greenberg, 1948). In all these experiments ¹⁴C and carrier serine were used. Our results show that in this conversion of glycine to serine, most or all of the nitrogen is retained. It thus appears that the reversible interconversion of glycine and serine is rapid compared with other metabolically reversible reactions in which the two amino-acids participate. One may also conclude that with both compounds oxidative deamination to the keto acid followed by reamination is slow. In fact, reamination of the keto acid may not occur at

all and the ^{15}N found in glycine after feeding ^{15}N -labelled amino-acids other than glycine and serine, may, as already suggested by Shemin (1946), arise by synthesis of glycine from a substance other than glyoxylic acid.

SUMMARY

1. Glycine containing excess ^{15}N was fed to rabbits and rats, and various amino-acids were isolated from protein of muscle and internal organs respectively. The glycine, serine, glutamic acid, arginine and tyrosine contained varying concentrations of isotopic nitrogen. Lysine and threonine had no excess ^{15}N .

2. It is concluded that threonine, like lysine, does not take part in the reversible transfer of nitrogen which is observed with other amino-acids, both 'essential' and 'non-essential'.

3. The glycine and serine isolated contained similar proportions of ^{15}N . This is taken to indicate that the conversion of glycine to serine is very fast compared with other reversible reactions in which the two amino-acids participate.

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The Oxidation of Various Synthetic α -Amino-acids by Mammalian D-Amino-acid Oxidase, L-Amino-acid Oxidase of Cobra Venom and the L- and D-Amino-acid Oxidases of *Neurospora crassa*

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Through the courtesy of Sir Charles Harington, F.R.S., National Institute for Medical Research, Hampstead (Elliott, Fuller & Harington, 1948; Elliott & Harington, 1949) and of Dr B. A. Hems, Glaxo Laboratories Ltd. (Elks, Hems & Ryman, 1948) we obtained twenty-seven synthetic α -mono-amino-acids not commonly available. The behaviour

of these substances towards D-amino-acid oxidase of sheep kidney, L-amino-acid oxidase of cobra venom and the L- and D-amino-acid oxidase of *Neurospora crassa* has been investigated. The rates of oxidation of the common amino-acids already examined by previous authors were also measured in order to obtain series of directly comparable data.