The Availability of the Acetyl Derivatives of Lysine for Growth

By A. NEUBERGER and F. SANGER (Benn W. Levy Student), *Biochemical Laboratory, Cambridge*

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The biological behaviour of the amino-acid lysine is abnormal in several respects. In a number of experiments with isotopic nitrogen and hydrogen Schoenheimer and his collaborators [Foster, Schoenheimer & Rittenberg, 1939; Schoenheimer, Ratner & Rittenberg, 1939 *a*, *b*; Weissman & Schoenheimer, 1941] have shown that, unlike most amino-acids, lysine does not accept N from other nitrogenous compounds, nor could deuterium be introduced metabolically into this molecule. It appears that even the first stages of lysine metabolism are irreversible. Until now no substances other than *l*-lysine have been found that will support the growth of young rats on a lysine-deficient diet. Berg [1936] has shown that *d*-lysine, which does not occur in nature, cannot replace the natural *l*-isomer. The α-hydroxy analogue of lysine is equally ineffective [McGinty, Lewis & Marvel, 1924]. All other α-hydroxy analogues of essential amino-acids that have been studied, with the exception of that of cystine, can replace the corresponding amino-acid in the diet. Gordon [1939] has shown that neither α-dimethyl lysine nor α-monomethyl lysine will support growth on lysine-deficient diets. Neither of the mono-acetyl derivatives of lysine has previously been synthesized, and it was thought it would be of interest to see if they can be converted to lysine in the body.

Acetyl amino-acids are of especial interest in connexion with the theory of Knoop [1910], which assumes that they may be intermediates in the synthesis of amino-acids from α-keto acids. du Vigneaud, Cohn, Brown, Irish, Schoenheimer & Rittenberg [1939] have recently revived this theory and produced additional evidence in support of it.

The deacetylation of α-acetyl-amino-acids appears to be a fairly general reaction in the body. The α-N-acetyl derivatives of *l*-tryptophan [du Vigneaud, Sealock & van Etten, 1932], and *dl*-phenylalanine [Carter, Handler, Binkley, Fishback, Risser & Weisiger, 1938], have been shown to replace the respective amino-acid in the diet and du Vigneaud, Loring & Craft [1934] have shown that diacetyl- and diformyl-cystine are converted to cystine in the body. Similarly acetyl-*l*-homocystine [du Vigneaud, Dyer & Jones, 1937] and formyl-*l*-methionine [Jackson & Block, 1933] can replace methionine in the diet. The results of Kimura [1929] also suggest the presence, in most organs, of an enzyme capable of deacetylating amino-acids. It thus seemed likely that the α-acetyl lysine, at least, would be converted to lysine in the body; however, here again, it was found that lysine behaves differently from other amino-acids.

METHODS

Chemical

Preparation of acetyl lysines. For the synthesis of the α- and ε-acetyl derivatives of lysine, use was made of the fact that in the copper complex of lysine the α-NH_2 and the COOH groups are bound in a stable chelate structure to Cu, whereas the ε-NH_2 group is free and can react with acylating reagents [Kurtz, 1938]. ε-acetyl lysine was prepared by direct acetylation of the Cu complex. α-acetyl lysine was prepared through ε-carbobenzoxy lysine. The latter had already been prepared by Bergmann, Zervas & Ross [1935]. It can, however, be much more conveniently prepared in about the same yield by treating the Cu complex with carbobenzoxy chloride. The product is quite insoluble, and after treatment with H_2S, ε-carbobenzoxy lysine can be isolated. This is acetylated in the α position, and the carbobenzoxy group removed by catalytic hydrogenation.

(*a*) *ε-N-acetyl-l-lysine.* Excess (basic) $CuCO_3$ was added gradually to a boiling solution of *l*-lysine sulphate (1·9 g.). After cooling, the solution was filtered. 1 equiv. of $Ba(OH)_2$ (1·7 g.) was then added, and the solution cooled in ice. Then acetic anhydride (1·2 g.) and $Ba(OH)_2$ (2·2 g.) were added in portions over 30 min. with shaking and cooling. After standing for 30 min. at room temperature, 0·34 ml. (0·63 g.) H_2SO_4 was added, and H_2S passed through the solution. The $BaSO_4$ and CuS were filtered off, and well washed by resuspending in boiling H_2O. The filtrate and washings were combined and concentrated to about 20 ml. and excess of Ba^{++} or $SO_4^{=}$ removed. The solution was evaporated to dryness, the residue dissolved in a minimum of hot H_2O and absolute ethanol added till there was a faint cloud. On cooling, the acetyl lysine crystallized in the form of flat plates. It was filtered off and washed with aqueous ethanol, absolute ethanol and ether. Yield 0·6 g. Another 0·4 g. could be obtained by working up the mother liquors, making a total yield of 1·0 g. or 55% of the theoretical value. (m.p. 249–253° (decomp.). $(\alpha)_D = 3{\cdot}4° \pm 0{\cdot}2$ ($c = 4{\cdot}0$). Found: C, 50·9; H, 8·7; N, 14·6%. Calc. for $C_8H_{16}O_3N_2$: C, 51·0; H, 8·5; N, 14·9%. Amino N (van Slyke): found: 7·48. Calc.: 7·45%.)

It formed an insoluble derivative with phosphotungstic acid, but no insoluble picrate.

(*b*) *ε-N-carbobenzoxy-l-lysine.* 1·8 g. *l*-lysine monohydrochloride was treated with excess $CuCO_3$ as above. 5 ml. 2*N* NaOH was added, and the dark blue solution cooled in ice. Carbobenzoxy chloride (2 ml.) and 2*N* NaOH (10 ml.) were

added in ten portions over 30 min. with shaking and cooling, care being taken not to allow the solution to become too alkaline. The Cu complex, which separated as a blue precipitate leaving a colourless solution, was filtered off and washed well with H_2O and ethanol. It was suspended in 200 ml. of H_2O, and H_2S passed in with mechanical shaking. The solution was then brought to the boil and filtered hot. On cooling, the ε-carbobenzoxy lysine crystallized in fine needles. After standing in the refrigerator overnight it was filtered off; a little more could be obtained by evaporating the mother liquors to a small volume. Yield was 80% of the theoretical. A sample recrystallized from H_2O showed $(\alpha)_D = 14{\cdot}4°$ ($c = 1{\cdot}6$) in two equivalents of HCl. This agrees with the value 14·0° found by Bergmann *et al.* [1935].

(*c*) *ε-N-carbobenzoxy-α-N-acetyl-l-lysine.* 2·2 g. ε-N-carbobenzoxy *l*-lysine were dissolved in 10 ml. *N* NaOH and cooled in ice. 1·5 ml. acetic anhydride and 20 ml. *N* NaOH were then added in ten portions over 30 min. with shaking and cooling. After standing at room temperature for 20 min. the solution was neutralized with HCl, and extracted with ether. The ether layer was discarded, and the aqueous layer made acid to congo red. This precipitated the ε-carbobenzoxy-α-acetyl lysine as an oil, which was extracted with ethyl acetate. The extract was dried and evaporated to dryness, leaving an oil which was used directly for the preparation of α-acetyl lysine. To obtain the ε-carbobenzoxy α-acetyl lysine in a crystalline form, the ethyl acetate solution was extracted with dilute $NaHCO_3$. This was then acidified and the oil again extracted into ethyl acetate. After drying, the ethyl acetate solution was evaporated to dryness, and the resulting clear oil crystallized on standing overnight. It could be recrystallized by dissolving in ethanol and adding H_2O gradually. The product which crystallized slowly did not melt sharply; m.p. 105–115°.

(*d*) *α-N-acetyl-l-lysine.* The dry oil obtained from 2·2 g. ε-carbobenzoxy lysine was dissolved in ethanol, and a drop of acetic acid and a few ml. H_2O were added. It was then hydrogenated in the usual way. The α-acetyl lysine, which usually crystallized from the filtered and concentrated solution directly, was taken up in a small volume of H_2O, which was then concentrated in a desiccator till it started to crystallize. Absolute ethanol was added to complete the separation. The yield of pure α-acetyl lysine was 0·8 g., and a further 0·1 g. was obtained by adding ether to the mother liquors, which made a total of 0·9 g. or 62% of the theoretical value from ε-carbobenzoxy lysine. The overall yield from *l*-lysine was 46%. (m.p. 250° (decomp.). $(\alpha)_D = 4{\cdot}7°$ ($c = 4{\cdot}4$). Found: C, 50·4; H, 8·6; N, 14·5%. Calc. for $C_8H_{16}O_3N_2$: C, 51·0; H, 8·5; N, 14·9%.)

Biological

Animals. Young, recently weaned male rats of a piebald type were used. They weighed 30–50 g. at the beginning of the experiment. In each experiment three groups, each of two to three animals, were used, each group being constituted of the littermates of the animals in the other two groups. One group received the basal diet alone, another the basal diet supplemented by pure lysine, while the third group was given the basal diet together with the substance under test. The control groups were necessary because of the variation in growth response to the addition of supplements, from litter to litter.

Diet. The basal lysine-deficient diet, which was fed *ad lib.*, was constituted as follows:

Starch	61 %	Lard	5 %
Gliadin	18 %	Cod-liver oil	1 %
Arachis oil	10 %	Salt mixture (McCollum)	5 %

In addition, each animal received daily a mixture containing 30 μg. aneurin, 30 μg. pyridoxin, 50 μg. riboflavin, 100 μg. Ca-*d*-pantothenate, 1 mg. nicotinic acid, 1 mg. inositol, 3 mg. choline chloride, and 0·15 ml. of an aqueous concentrate of whole liver, corresponding to 2 g. fresh tissue.

RESULTS

Effect of oral administration of amino-acids

Rats fed on the basal diet alone failed to grow, but if 40 mg. *l*-lysine monohydrochloride were added to the daily ration the animals grew at the rate of 1–2 g./day, showing that lysine deficiency was the factor which limited growth. Addition of more than 40 mg./day of *l*-lysine monohydrochloride had no further effect on the growth rate.

The analytically pure acetyl lysines were fed at the rate of 42 mg./day, which is equivalent to a daily supplement of 40 mg. *l*-lysine monohydrochloride. The rats entirely failed to grow with oral α-acetyl lysine (Fig. 1), whereas with lysine they

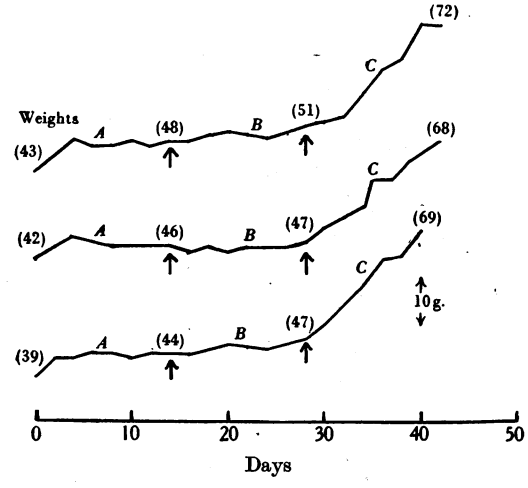

Fig. 1. The availability of α-N-acetyl-*l*-lysine for growth. The arrows indicate a change of diet. The weights of the animals at the beginning and end of each feeding period are shown in brackets. The diets fed to the animals were as follows: *A*, basal 18% gliadin diet; *B*, basal diet + 42 mg. α-N-acetyl-*l*-lysine/day; *C*, basal diet + 40 mg. *l*-lysine monohydrochloride/day.

grew about 1·5 g./day. This clearly shows that α-acetyl lysine cannot effectively replace lysine in the diet.

On the other hand, the growth rate with 42 mg. ε-acetyl lysine was almost as great as with lysine

(Fig. 2), and when 84 mg. were fed, the growth was equal to that with lysine; thus ε-acetyl lysine can be converted to lysine in the body to a considerable extent, although probably not quantitatively.

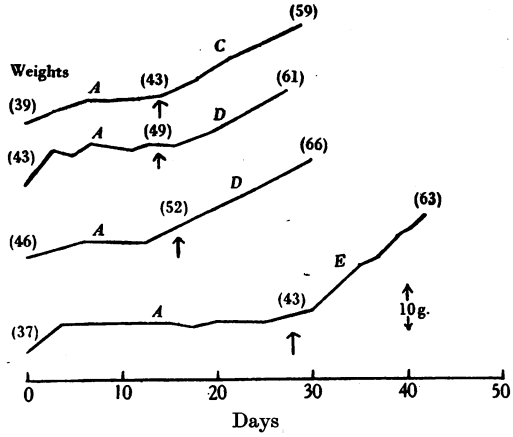

Fig. 2. The availability of ε-N-acetyl-*l*-lysine for growth. The arrows indicate a change of diet. The weights of the animals at the beginning and end of each feeding period are shown in brackets. The diets fed to the animals were as follows: *A*, basal 18% gliadin diet; *C*, basal diet + 50 mg. *l*-lysine monohydrochloride/day; *D*, basal diet + 42 mg. ε-N-acetyl-*l*-lysine/day; *E*, basal diet + 84 mg. ε-N-acetyl-*l*-lysine/day.

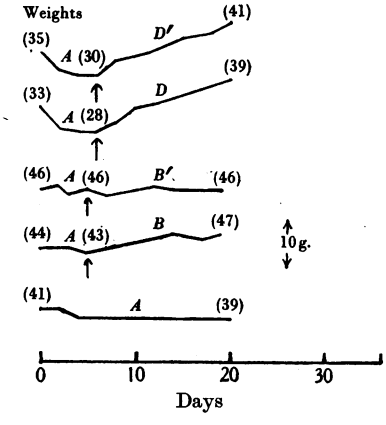

Fig. 3. The effect of intraperitoneal injection of the acetyl lysines. The arrows indicate a change of diet. The weights of the animals at the beginning and end of each feeding period are shown in brackets. The diets were as follows: *A*, basal 18% gliadin diet; *B*, basal diet + 42 mg. α-N-acetyl-*l*-lysine (fed)/day; *B'*, basal diet + 21 mg. α-N-acetyl-*l*-lysine (injected twice daily); *D*, basal diet + 42 mg. ε-N-acetyl-*l*-lysine (fed)/day; *D'*, basal diet + 21 mg. ε-N-acetyl-*l*-lysine (injected twice daily).

Effect of parenteral administration of amino-acids

It appeared possible that the reason why α-acetyl lysine did not effectively replace lysine was its failure to be absorbed from the gut. Furthermore, ε-acetyl lysine may have been able to replace lysine because it was hydrolyzed to lysine by bacteria in the intestine. The acetyl lysines were therefore injected intraperitoneally into rats, any changes that may take place in the gut thus being avoided. Conrad & Berg [1937] have shown that when lysine is fed in one dose each day it gives the same growth response as when it is fed in half the dose twice daily; they also showed that better results are obtained if the daily dose is given in two injections at 12 hr. intervals. In our experiments the rats fed on the basal diet were given two daily injections of a solution containing 21 mg. acetyl lysine. The results (Fig. 3) confirm those of the feeding experiments, and thus show that the differences then observed were not due to any reaction which may take place in the gut.

DISCUSSION

The feeding experiments demonstrate that for the young growing rat, ε-acetyl lysine can replace *l*-lysine in the diet; this is presumably due to its conversion into lysine by deacetylation to give its parent amino-acid. α-Acetyl-*l*-lysine, however, is not appreciably converted. This may be due to its not being broken down at all. This seems unlikely since other α-N-acetyl-amino-acids can be metabolized very readily. A more likely explanation is that it is oxidized, presumably at the ε-NH_2, before deacetylation can take place, whereas with the ε-acetyl compound deacetylation takes place more readily than oxidation of the α-NH_2. If we assume that the rate of deacetylation is about the same in both cases, then it would appear that the ε-NH_2 is more readily oxidized than the α-NH_2, and this may be true for lysine itself, which suggests that the initial breakdown of lysine may take place at the ε-NH_2 and not at the α-NH_2 group. However, it is probably unwise to compare the metabolism of the acetyl-lysines, in which one NH_2 is masked, with that of lysine itself, since the action of enzymes will probably be affected by the presence of additional free NH_2 groups.

The results with α-acetyl lysine are of interest in connexion with the acetylation theory of Knoop [1910]. Since α-acetyl lysine, the assumed intermediate in lysine synthesis, cannot be converted into lysine, this would explain the fact that lysine cannot be synthesized in the body from the corresponding hydroxy or keto-acid, and that N^{15} cannot enter the α-NH_2 of lysine. However, it was not suggested by Knoop, and it seems unlikely, that acetylation combined with reductive amination is the only method of amino-acid synthesis; but our results do seem to lend support to the theory.

SUMMARY

1. Methods are described for the synthesis of ε-carbobenzoxy lysine, α-N-acetyl lysine and ε-N-acetyl lysine from the copper complex of lysine.

2. ε-N-acetyl-*l*-lysine is available for the growth of rats on a lysine deficient diet, whereas α-N-acetyl-*l*-lysine is not so available.

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The Thermal Decomposition of Aneurin and Co-carboxylase at Varying Hydrogen Ion Concentrations

By R. G. BOOTH, *Cereals Research Station, Ministry of Food, Old London Road, St Albans*

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Molitor & Sampson [1936] have stated that pure aneurin (vitamin B_1 hydrochloride) in aqueous solution at *p*H 3·5 may be heated to 120° without undergoing decomposition. Foodstuffs containing aneurin are rarely if ever prepared, cooked and eaten in such an acid condition, and this observation, therefore, is nutritionally of little practical importance. Farrer [1941] has made an extensive study of the effect of *p*H's ranging from 3 to 9 upon the destruction of aneurin in solution at 100° and finds that even at *p*H 3 there is 16 % loss after 1 hr. and 29 % after 3 hr. It may therefore be that assay methods were not sufficiently advanced at the time of publication of Molitor & Sampson's work to indicate with certainty the small loss which it would appear probable had taken place. On the other hand, as will be discussed more fully later, the buffer used by Farrer may have contained impurities which catalyzed the destruction which was noted. Beadle, Greenwood & Kraybill [1943] have verbally presented details of a study similar to that of Farrer, but a full account of their work has only just come to hand.

Apart from these references to rate of destruction under specific and carefully controlled conditions and certain early studies [Chick & Hume, 1917; Sherman & Grose, 1923; Sherman & Burton, 1926; Guha & Drummond, 1929] there is little factual information in the literature on the subject apart from statements concerning losses on baking bread and in cooking a few other foodstuffs. In view of the fact that the fortification of foodstuffs with vitamins and other dietary essentials is being widely carried out (in respect of vitamin B_1 and other factors) it was considered desirable to determine such losses over a fairly wide range of conditions (but in the absence of oxidizing or reducing agents) which would serve to indicate the magnitude of loss to be expected in any non-pressure cooking or preparatory process (e.g. blanching) applied to foodstuffs.

While wheat and some other cereals contain vitamin B_1 as hydrochloride only, in other vegetable (e.g. soya) and most animal products B_1 activity resides in the pyro-phosphoric ester (co-carboxylase) and/or monophosphate of the vitamin in addition