Enzymatic Resolution of Racemic Amino Acids

Part VII. Availability of the Acyl Derivatives of Lysine for the Growth of Rats

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The present paper is concerned with the availability of the acyl derivatives of lysine for the growth of young rats in the course of studying the enzymatic resolution of DL-lysine with mold acylase. The enzymatic resolution of DL-lysine to optically-active L and D-isomers was carried out in either of the following two ways, namely, the asymmetric hydrolysis of diacetyl-DL-lysine or that of ϵ -benzoyl- α -acetyl-DL-lysine.

The oral administration of ϵ -acetyl-L-lysine to rats fed on the lysine-deficient diet supported the growth of young rats at a rate approximately two-thirds of that observed when L-lysine was supplied. ϵ -Benzoyl-L-lysine proved to be quite ineffective while diacetyl lysine showed a slight but insignificant increase in body weight.

Neuberger and Sanger¹⁾ showed that ϵ -acetyl-L-lysine can replace the free L-lysine when fed to growing rats resulting in good growth as those subjected to the L-lysine diet by oral administration or by intraperitoneal injection. Paik and Greenstein²⁾ et al., have recently reported that supplementation of the lysine-deficient diet with ϵ -acetyl-L-lysine leads to the growth of rats which is in accord with the earlier observations of former investigators and have proved the presence of an ϵ -acylase in the rat tissues, especially in the kidney, capable of hydrolytic deacetylation of ϵ -acetyl-L-lysine which is distinct from the renal α -acylase.

The present authors^{3,4,5)} have been engaged in a series of studies on enzymatic resolution of racemic amino acids by using mold amino acid acylase. The present paper concerns the enzymatic resolution of DL-lysine and the availability of ϵ -acyl derivatives of L-lysine for the growth response in rats. Acyl deriva-

tives of L-lysine and L and D-isomers of lysine were obtained by enzymatic resolution procedure with the mold acylase of *Penicillium* as described previously.^{4,5)} The resolution of DL-lysine was undertaken in either of the following two ways, namely, by asymmetric hydrolysis of diacetyl-DL-lysine or that of ϵ -benzoyl- α -acetyl-DL-lysine. In these procedures ϵ -acetyl-L-lysine and ϵ -benzoyl-L-lysine were obtained as intermediate products.

EXPERIMENTAL

I. Resolution of Diacetyl-DL-lysine

Diacetyl-DL-lysine. Diacetyl-DL-lysine was prepared as usual, namely, by acetylation of DL-lysine·2HCl (0.1 Mol) in 2N NaOH (0.3 Mol) with acetic anhydride (0.4 Mol) and the simultaneous addition of 2N NaOH (0.4 Mol) in an ice-bath. Diacetyl-DL-lysine was crystallized from an alcohol-acetone mixture. Yield 80% of theo., m. p. 141°C.

Anal. Found: N, 11.87. Calcd. for $C_{10}H_{18}O_4N_2$: N, 12.10%.

ϵ -Acetyl-DL-lysine. Fifteen g of diacetyl-DL-lysine was dissolved in 400 ml of water and neutralized by the addition of 3.5 g of calcium carbonate. The solution was incubated with the addition of *Penicillium* acylase which was prepared from 300 g of molded bran as described previously.⁴⁾ After two days' incubation,

1) A. Neuberger and F. Sanger, *Biochem. J.*, **37**, 515 (1943).

2) W. K. Paik, L. Bloch-Frankenthal, S. M. Birnbaum, M. Winitz and J. P. Greenstein, *Arch. Biochem. Biophys.*, **69**, 56 (1957).

3) K. Michi and H. Nonaka, *This Bulletin*, **19**, 153 (1955).

4) K. Michi and H. Tsuda, *This Bulletin*, **21**, 18 (1957).

5) K. Michi and H. Tsuda, *This Bulletin*, **21**, 235 (1957).

the reaction mixture was treated with oxalic acid so as to remove calcium. The filtrate was evaporated in vacuo to a small volume and treated with an excess of hot absolute alcohol. ϵ -Acetyl-L-lysine was crystallized out rapidly from the solution. After being allowed to stand in a refrigerator overnight, the precipitate was filtered and washed with absolute alcohol. Another crop of ϵ -acetyl-L-lysine was obtained from the filtrate. Yield 5.5 g (90 % of theo.) Recrystallized compound from dilute alcohol gave m. p. 244°C, $[\alpha]_D^{25} + 22.0^\circ$ (c 2, 5N HCl).

Anal. Found: N, 14.62. Calcd. for $C_8H_{16}O_3N_2$: N, 14.89 %.

L-Lysine. ϵ -Acetyl-L-lysine (5 g) added with 2N HCl was refluxed for two hours. L-Lysine-2HCl was obtained from the evaporated residue of the digest. Yield 4.9 g (84.5 % of theo., over-all yield 70% from diacetyl lysine). Recrystallized L-lysine-2HCl gave m.p. 195–198°C, $[\alpha]_D^{18} + 17.26^\circ$ (c 4, 5N HCl), $[\alpha]_D^{19} + 25.86^\circ$ (for free L-lysine).

Anal. Found: N, 12.52. Calcd. for $C_6H_{16}O_2N_2Cl_2$: N, 12.77 %.

D-Lysin. The filtrate of ϵ -acetyl-L-lysine and the washings were evaporated to dryness and dissolved in 50 ml of water. The solution which possessed a ninhydrin reaction was adjusted with 0.5 ml of 6N HCl to pH 1 and passed through a 20 cm column of Dowex 50×4 (Na^+) in order to remove ϵ -acetyl-L-lysine. The filtrate which gave a negative ninhydrin reaction was acidified with 6N HCl to a 2N HCl content and refluxed for two hours. When the digest was evaporated to dryness in vacuo, D-lysine-2HCl was obtained with the addition of alcohol. Yield 5 g (70 % of theo.) The recrystallized compound gave m. p. 195–198°C and $[\alpha]_D^{18} - 17.20^\circ$ (c 4, 6N HCl).

II. Resolution of ϵ -Benzoyl- α -acetyl-DL-lysine

ϵ -Benzoyl- α -acetyl-DL-lysine. ϵ -Benzoyl- α -acetyl-DL-lysine was prepared by acetylation of ϵ -benzoyl-DL-lysine with acetic anhydride and 2N NaOH as usual. The separated oily ϵ -benzoyl- α -acetyl-DL-lysine obtained from the reaction mixture by the addition of 6N HCl was brought to crystallization in a refrigerator. The compound which was collected on filter by suction was washed with cold water. Recrystallization was carried out by dissolving the compound in dilute sodium hydroxide in a cold bath and precipitation with the addition of 2N HCl. The purified compound gave m. p. 145°C.

Anal. Found: N, 9.45. Calcd. for $C_{15}H_{20}O_4N_2$: N, 9.58 %.

ϵ -Benzoyl-L-lysine. ϵ -Benzoyl- α -acetyl-DL-lysine (60 g) was dissolved in water added with 2N NaOH. The solution which showed pH 7 was incubated with *Penicillium* acylase prepared from 800–1000 g of molded bran at 37°C for two days. ϵ -Benzoyl-L-lysine precipitated during incubation was filtered and washed with cold water and then with alcohol. Yield 22 g (85 % of theo.) m.p. 266°C, $[\alpha]_D^{18} + 19.48^\circ$ (C 2.4, 6N HCl).

The filtrate was separated for the preparation of D-lysine.

L-Lysine. ϵ -Benzoyl-L-lysine (20 g) was refluxed with 6N HCl (200 ml) for eight hours. Yield 14 g (80 % of theo.) Recrystallized L-lysine-2HCl gave m.p. 198°C and $[\alpha]_D^{18} + 17.46^\circ$ (C 4, 6N HCl), $[\alpha]_D^{19} + 26.07^\circ$ (for free base).

Anal. Found: N, 12.47; Cl, 32.30 %. Calcd. for $C_6H_{16}O_2N_2Cl_2$: N, 12.77; Cl, 32.39 %.

D-Lysine. The filtrate separated from ϵ -benzoyl-L-lysine was evaporated to about 70 ml and the solution acidified with 2N HCl was extracted with ethylacetate as described by Chibata et al.⁶⁾ The ethylacetate layer was separated from the ninhydrin reaction-positive solution. The combined ethylacetate solution was evaporated and the residue was refluxed with 6N HCl (200 ml) for eight hours. The purified D-lysine-2HCl gave m. p. 198°C and $[\alpha]_D^{18} - 17.38^\circ$ (C 4, 6N HCl), $[\alpha]_D^{19} - 26.04^\circ$ (for free base). Yield 11g (63% of theo.)

Anal. Found: N, 12.42; Cl, 32.43 %. Calcd. for $C_6H_{16}O_2N_2Cl_2$: N, 12.77; Cl, 32.39.

III. Animal Experiment.

Male weanling rats of the Wistar strain with an average weight of 50 to 55 g were used. The animals were divided into seven groups, each consisting of six to eight rats. The animals were housed in individual cages equipped with raised screen bottoms. They were fed ad libitum and weighed twice a week during the experimental periods of four weeks.

As shown in Table I, the basal lysine deficient diet (Diet I) consisted of wheat flour, Yakifu (dried gluten), soybean oil, salts and known vitamins. The rations were supplemented with 0.6 % of DL-lysine (Diet II), 0.3 % of L-lysine (Diet III), 0.3 % of D-lysine (Diet IV), 0.258 % of ϵ -acetyl-L-lysine (Diet V), 0.342 % of ϵ -benzoyl-L-lysine (Diet VI) and 0.63 % of diacetyl-DL-lysine (Diet VII) in equivalents to a supplement of L-lysine and DL-lysine. The nitrogen content of the rations was maintained at the same level by appropriate addition of glycine and a small amount of glucose

6) I. Chibata, S. Yamada and S. Yamada, This Bulletin, 20, 174 (1956).

TABLE I COMPOSITION OF DIETS

Ingredient	Diet I	Diet II	Diet III	Diet IV	Diet V	Diet VI	Diet VII
Wheat flour ¹⁾	84 %	84 %	84 %	84 %	84 %	84 %	84 %
Yakifu ²⁾	6	6	6	6	6	6	6
Salt mixture	4	4	4	4	4	4	4
Soybean oil	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Vitamin A, D, E oil ³⁾	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Choline chloride	0.15	0.15	0.15	0.15	0.15	0.15	0.15
DL-Lysine·2HCl		0.6					
L-Lysine·2HCl			0.3				
D-Lysine·2HCl				0.3			
ϵ -Acetyl-L-lysine					0.258		
ϵ -Benzoyl-L-lysine						0.342	
Diacetyl-DL-lysine							0.63
Glycine	0.42		0.21	0.21	0.21	0.21	
Glucose	0.43	0.25	0.34	0.34	0.38	0.30	0.22
	⁴⁾ 100.00	100.00	100.00	100.00	100.00	100.00	100.00

1) N, 1.48 %.

2) Commercial wheat gluten, N, 5.31 %.

3) 2000 I. U. of vitamin A, 400 I. U. of vitamin D and 10 mg of vitamin E contained in 0.5 g of oil.

4) Calculated total N % of amino acids and protein: N, 1.64 % in 100 g of diet.

TABLE II VITAMIN SUPPLEMENTS

	mg per kg of diet
Thiamine hydrochloride	5
Riboflavin	10
Pyridoxine hydrochloride	5
Nicotinic acid	25
Calcium pantothenate	25
<i>p</i> -Aminobenzoic acid	300
2-Methyl-1, 4-naphthoquinone	2
Biotin	0.1
Folic acid	0.2
Vitamin B ₁₂	0.02
Inositol	100

nutritive value of wheat flour protein could be improved by the addition of lysine together with other essential amino acids. Sure⁷⁾ has shown that the quality of milled wheat flour (86.4 %) diet is improved by the supplement of 0.4 % of L-lysine, 0.2 % of DL-threonine and 0.7 % of DL-valine. The present authors have employed a wheat flour diet supplemented with dried gluten (Yakifu) as a substitute for supplementation with pure essential amino acids, such as DL-threonine.

As shown in Table III, the growth response

TABLE III GROWTH RESPONSES OF YOUNG RATS FED ON EXPERIMENTAL DIETS

Diet No.	No. of animals	Mean gain in weight in a 28-day period and probable error	Nature of diet
I	8	10.6 $\pm$ 1.37	Negative control
II	7	48.4 $\pm$ 3.30	DL-Lysine diet
III	6	45.7 $\pm$ 3.54	L-Lysine diet
IV	7	10.9 $\pm$ 1.89	D-Lysine diet
V	6	31.7 $\pm$ 1.49	ϵ -Acetyl-L-lysine diet
VI	6	10.8 $\pm$ 1.16	ϵ -Benzoyl-L-lysine diet
VII	8	14.3 $\pm$ 1.16	Diacetyl-DL-lysine diet

was added to make the contents up to 100 %.

RESULTS AND DISCUSSION

Several investigators have shown that the

in rats supplemented with L-lysine, DL-lysine and ϵ -acetyl-L-lysine was definitely improved over that of the negative control group (Diet

7) B. Sure, *J. Nut.*, **50**, 235 (1953).

I). On the L-lysine diet the average growth rate was $45.7\text{ g}\pm 3.5$ for four weeks or a 1.6 g gain per day, on the DL-lysine diet $48.4\text{ g}\pm 3.3$ for four weeks or a 1.7 g gain per day and on the ϵ -acetyl-L-lysine diet $31.7\text{ g}\pm 1.5$ for four weeks or a 1.1 g gain per day. Paik et al. have recently reported a similar growth response in rats by employing a more complete chemically defined-diet. Animals fed on the D-lysine diet and ϵ -benzoyl-L-lysine diet showed approximately the same average gains, $10.9\text{ g}\pm 1.9$ and $10.8\text{ g}\pm 1.2$, respectively, and actually no improvement in growth was observed when compared with $10.6\text{ g}\pm 1.4$ of the control group without supplement. Those fed on diacetyl-DL-lysine diet showed a slight but insignificant increase in body weight.

The availability of ϵ -acetyl-L-lysine for the growth of rats was confirmed to be in accord with the results of American and English investigators. ϵ -Acetyl-L-lysine is not quite so effective in replacing L-lysine in the diet so well as described by Paik et al. Although ϵ -lysine

acylase and α -amino acid acylase have been found in the tissues of rat by Greenstein et al., diacetyl-DL-lysine almost failed to improve the lysine-deficient diet. However, a slight increase of body weight over that of the lysine-deficient group exhibits a very slow inversion of diacetyl-DL-lysine to L-lysine. ϵ -Benzoyl-L-lysine was quite ineffective in replacing L-lysine. It is supposed that the derivative is not absorbed by the alimentary tract because of its insolubility in water or digestive juice. Optically-active L-lysine had the best availability and D-lysine was completely ineffective.

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