Utilization of Ethyl Esters of α -Keto Analogues of Isoleucine, Leucine and Valine for Growth in Rats

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It has been established by earlier investigations that the α -keto analogues of methionine, phenylalanine, tryptophan, histidine, isoleucine, leucine and valine can replace these amino acids in the diet. It has also been shown¹ that the isopropyl ester of both L- and D-phenylalanine is able to produce growth in rats. It therefore appeared probable that amino acids could also be replaced by the esters of their α -keto analogues. In order to ascertain whether this is the case, the experiments reported in the following were made with the ethyl esters of α -ketoisovaleric acid, α -ketoisocaproic acid and α -keto- β -methyl-valeric acid. Strangely enough, it was found that the ethyl esters of these keto acids were poorly utilized, and that they were unable to produce growth in young rats.

EXPERIMENTAL

The ethyl esters of α -ketoisovaleric acid, α -ketoisocaproic acid and α -keto- β -methyl-valeric acid were produced as follows:

Ethyl- α -alkylacetoacetate (III) was synthesized from the corresponding alkyl bromide and ethylacetoacetate, using a method similar to that of Rupe and Courvoisier² for the preparation of ethyl- α -isopropylacetoacetate. The ethyl esters of α -oxamino acid and α -keto acid were obtained with the method described by Sen³ for the synthesis of ethyl- α -ketoisovalerate.

Ethyl- α -isopropylacetoacetate (III). 1 300 g of ethylacetoacetate are added to a solution of sodium alcoholate, obtained from 4.5 liters of absolute alcohol and 230 g of sodium. While stirring, the solution is heated to boiling and 340 g of NaI are added; 1 350 g of isopropyl bromide are then added in the course of two hours. The solution is refluxed over night. After cooling, the solution is filtered. The alcohol is distilled off *in vacuo* and 1 000 ml of water are added to the residue. The solution is then extracted three times, each time with 1 000 ml of ether, and the combined ether extracts are washed twice with 250 ml of water each time. After drying with Na_2SO_4 , the ether extract is distilled *in vacuo*; the fraction distilling between 93–94.5° C at 18 mm contains the ethyl ester of isopropylacetoacetic acid. Yield: 49 % (850 g).

Ethyl- α -oxaminoisovalerate (IV). 425 g of ethyl-*isopropylacetoacetate* are added to a sodium alcoholate solution, prepared from 850 ml of absolute alcohol and 57 g of sodium. Ethyl nitrite, which has been dried over CaCl_2 , is bubbled into this mixture. An ice bath is used to prevent the temperature of the solution from exceeding 40° C. The ethyl nitrite is bubbled through until the formation of heat has ceased. After the solution has stood over night at room temperature, the alcohol is removed *in vacuo*. To the residue, which consists of the sodium salt of ethyl- α -oxaminoisovalerate, dilute sulphuric acid (1 : 3) is added with cooling until an acid reaction is obtained. Extraction with ether is then performed three times, with 100 ml each time. After drying over Na_2SO_4 , the ether is removed, and the fraction is taken which distills between 128–131° C at 12 mm. Yield: 60 % (270 g).

Ethyl- α -ketoisovalerate (V). 250 g of ethyl- α -oxaminoisovalerate are dissolved in 500 ml of 85 % formic acid. The solution is cooled to 0° C. 150 g of nitrosyl sulphate are added in small portions, at such a rate that the temperature does not exceed 5° C. When the reaction is complete, the solution is allowed to stand for one hour at room temperature, when it usually turns from greenish-blue to yellow. To this mixture are added 1 500 ml of ice water and the solution is extracted three times, with 500 ml of ether each time. The ether is removed *in vacuo* (20 mm Hg); the formic acid being removed at the same time. The residue is taken up in 500 ml of ether and extracted with 20 % Na_2CO_3 , in order to remove the free α -ketoisovaleric acid. After drying over Na_2SO_4 , the ether solution is fractionated; the fraction distilling at 72° C at 16 mm contains ethyl- α -ketoisovalerate. After redistillation, 100 g are obtained, corresponding to a yield of 40 %. (Found: C 58.0; H 8.4. Calc. for $\text{C}_7\text{H}_{12}\text{O}_3$: C 58.3; H 8.4).

Ethyl- α -ketoisocaproate and ethyl- α -keto- β -methyl-valerate are prepared in a similar way to ethyl- α -ketoisovalerate, except that 1 500 g of isobutyl bromide and *sec*-butyl bromide, respectively, are used for the synthesis of ethylisobutylacetoacetate (III) and ethyl-*sec*-butylacetoacetate (III). For the preparation of ethyl- α -oxaminoisocaproate (IV) and ethyl- α -oxamino- β -methyl-valerate (IV), 460 g of ethylisobutylacetoacetate and ethyl-*sec*-butylacetoacetate, respectively, are used. For the synthesis of ethyl- α -ketoisocaproate (V) and ethyl- α -keto- β -methyl-valerate (V), 280 g of ethyl- α -oxaminoisocaproate and ethyl- α -oxamino- β -methyl-valerate, respectively, are taken. On synthesis, the yields are as follows:

a) ethylisobutylacetoacetate	43–47 %
ethyl- <i>sec</i> -butylacetoacetate	43 »
b) ethyl- α -oxaminoisocaproate	27 »
ethyl- α -oxamino- β -methyl-valerate	56 »
c) ethyl- α -ketoisocaproate	41 »
ethyl- α -keto- β -methyl-valerate	36 »

Ethyl- α -ketoisocaproate is redistilled *in vacuo* at 93° C at 25 mm. (Found: C 61.1; H 9.2. Calc. for $\text{C}_8\text{H}_{14}\text{O}_3$: C 60.7; H 8.9).

Ethyl- α -keto- β -methyl-valerate is redistilled *in vacuo* at 84–88° C at 21 mm. (Found: C 60.7; H 8.9. Calc. for $\text{C}_8\text{H}_{14}\text{O}_3$: C 60.7; H 8.9).

Table 1. Composition of the experimental diets.

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8
	g	g	g	g	g	g	g	g
Sucrose	604.5	629.5	603.5	639.5	601.5	619.5	563.5	599.5
Sucrose-vitamin mixture (See Table 2)	150	150	150	150	150	150	150	150
Cellulflour	20	20	20	20	20	20	20	20
Salt mixture *	40	40	40	40	40	40	40	40
Cod liver oil	5	5	5	5	5	5	5	5
Wheat germ oil	15	15	15	15	15	15	15	15
Inositol	1	1	1	1	1	1	1	1
Choline chloride	2	2	2	2	2	2	2	2
DL-Histidine	5	5	5	5	5	5	5	5
DL-Isoleucine	40	40	—	—	40	40	40	40
DL-Leucine	20	20	20	20	—	—	20	20
DL-Lysine hydrochloride	22.4	22.4	22.4	22.4	22.4	22.4	22.4	22.4
DL-Methionine	5	5	5	5	5	5	5	5
DL-Phenylalanine	20	20	20	20	20	20	20	20
DL-Threonine	10	10	10	10	10	10	10	10
DL-Tryptophan	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8
DL-Valine	—	—	30	30	30	30	30	30
Sodium bicarbonate	11.3	11.3	11.3	11.3	11.3	11.3	11.3	11.3
Ethyl- α -ketoisovalerate	25	—	—	—	—	—	—	—
Ethyl- α -keto- β -methyl- valerate	—	—	36	—	—	—	36	—
Ethyl- α -ketoisocaproate	—	—	—	—	18	—	—	—
	1 000	1 000	1 000	1 000	1 000	1 000	1 000	1 000

* "No. 12", Jones and Foster 1942⁶.

White male rats aged from 30 to 40 days were used for the experiments. The animals were kept in individual cages and allowed to eat and drink *ad libitum*. The cages were placed in a room with a constant temperature of 26–27° C. During the two days immediately preceding the experiments, the animals were given a diet containing the same ingredients as the experimental diet, except that 18 % of an enzymatic casein hydrolysate (Aminosol Vitrum) was substituted for the amino acids. This was in order to avoid the changes in weight which otherwise occur when animals are transferred from one type of diet to another.

The diets investigated are listed in Table 1. All the diets contained the racemic form of the essential amino acids in the same concentrations as in earlier experiments ⁴.

Diets 1 and 2 were used to study the effect on growth of the ethyl ester of α -ketoisovaleric acid. Diet 1 contained the ethyl ester of α -ketoisovaleric acid instead of valine, and both valine and its ethyl ester were omitted from diet 2. The quantity of the ester amounted to 2.5 %, corresponding to 2 % of α -ketoisovaleric acid, which had been found in earlier experiments ⁴ to produce satisfactory growth in rats.

Both diets 3 and 4 were devoid of isoleucine. Diet 3 contained 3.6 % of ethyl- α -keto- β -methyl-valerate. The concentration of the ester exceeded the theoretically calculated quantity of L-leucine (1 %) in diets 1 and 2 by 50 %. The larger quantity of the ester than that calculated was given in order to compensate for a possibly poor yield on hydrolysis and amination.

For the investigation of the ethyl ester of α -ketoisocaproate, diets 5 and 6 were prepared. The concentration of the ester in diet 5 was 50 % greater than the calculated quantity, for the same reason as in diet 3.

Since the ethyl esters have an unpleasant smell, it was anticipated that the animals might not eat sufficient quantities. Consequently, a diet was composed which contained both ethyl- α -keto- β -methyl-valerate and 4 % of DL-isoleucine (diet 7). The growth produced with this diet was compared with that produced by diet 8, which contained all the essential amino acids.

The first series comprised two groups with five rats in each. One group was given diet 1 and the other diet 2. In the second series, a similar comparison was made between diets 3 and 4, and in the third series between diets 5 and 6. The results are recorded in Fig. 1.

The comparison between the effect of diets 7 and 8 was made as follows. Two groups with five rats in each were given diet 3 and diet 4, respectively, for 12 days. After this time they were given the same diet with the addition of 4 % of DL-isoleucine (diets 7 and 8). The effect of these diets on the body weight is shown in Fig. 2.

A further series of experiments was made, using groups of five rats for tests of each of the diets. The investigations covered a 10-day period. In Table 3, the means and the standard errors of the means are given both for the combined groups, each including 10 rats on the experimental diet for 10 days, and for the groups in which the experiments were continued for a longer period. On the basis of these figures, calculations can be made of the possible significant difference between the results in the different groups in the individual series.

If all the ester were converted into the respective amino acid in such a way that it could be utilized for protein synthesis, growth would be maximal. Under the experimental conditions in question, this would amount to an increase in weight of 12 ± 0.5 g per 10 days⁵. By interpolating the results of earlier experiments, an approximate figure can be given for the concentration of L-valine, L-isoleucine and L-leucine, respectively, that would produce the changes in weight obtained during the first 10 days, as recorded in Table 3. In these calculations, curves were drawn on the basis of the difference in the change in weight with and without 0.5 % of the corresponding DL-amino acid, *i. e.*, 0.25 % of the biologically active L form (Wretling⁶, Table IX). From these curves, a value was obtained for the quantity of L-amino acid required to produce the difference in the change in body weight obtained with the various diets, *i. e.*, those with and without the ethyl ester of the keto acid of the respective amino acids (Table 3).

RESULTS AND DISCUSSION

It is evident from Fig. 1 that when a diet lacking one of the amino acids valine, isoleucine or leucine was supplemented with the ethyl ester of the corresponding keto acid, it was unable to produce growth in young rats. The curves nevertheless suggest that some proportion of the relevant ethyl ester was utilized, since the loss of weight was not as marked as in the absence of this ester. This applies in particular to the ethyl ester of ketoleucine (α -ketoisocaproic acid) and of ketoisoleucine (α -keto- β -methyl-valeric acid). It may also be inferred from Table 3 that there is a significant difference between the loss of weight after 10 days in a comparison between the diets with and without the ethyl ester of ketovaline (α -ketoisovaleric acid) — ($p = 0.01$), the ethyl ester of ketoisoleucine ($p = 0.001$) and the ethyl ester of ketoleucine ($p = 0.001$). After 18 and 25 days as well, a significant difference is present between the changes in weight with and without the ethyl ester of ketoisoleucine and ketoleucine, respectively. On the other hand, no significant difference is noted after 18 days with and without the ethyl ester of ketovaline. A possible explanation is that only two of the animals given diet 2 (without valine and its ethyl ester) survived; consequently, no reliable statistical calculations could be made. The fact that 3 out of the 5 animals on this diet died, but none of those given the ethyl ester of ketovaline, nevertheless affords

Fig. 1. Changes in weight in growing rats given diets without valine, leucine and isoleucine and diets in which these amino acids were replaced by the ethyl esters of the corresponding α -keto acids. Each curve represents the mean value for 5 rats. The figures in brackets at the beginning and end of each curve denote the mean initial and final weight, respectively.

- Series 1. { Curve I. Diet 1: without valine, with ethyl- α -ketoisovalerate.
 { Curve II. Diet 2: without valine.
 Series 2. { Curve III. Diet 3: without isoleucine, with ethyl- α -keto- β -methyl-valerate.
 { Curve IV. Diet 4: without isoleucine.
 Series 3. { Curve V. Diet 5: without leucine, with ethyl- α -ketoisocaproate.
 { Curve VI. Diet 6: without leucine.

additional evidence that part of the ethyl ester was converted into L-valine. Thus, it both reduced the loss of weight and prolonged the survival time.

The results obtained show that the ethyl esters of ketovaline, ketoisoleucine and ketoleucine cannot be converted by the organism into the respective amino acids to a sufficient extent to promote growth. It is, however, also apparent from the experiments that a small part of the esters is converted into the corresponding amino acids, since the loss of weight is less than without the relevant ester. It may be inferred from the approximative calculations incorporated in Table 3 that the reduction in the loss of weight corresponds to a content of 0.1 % of L-valine, 0.2 % of L-isoleucine and 0.1 % of L-leucine, respectively, in the diet. Theoretically, the concentrations of the ethyl esters in the diet correspond to 2 % of L-valine, 3 % of L-isoleucine and 1.5 % of L-leucine. It may therefore be concluded that only 5 to 7 % of the esters supplied is utilized.

Fig. 2. The effect on growing rats of the addition of isoleucine to the diet; both diets had earlier been devoid of isoleucine, but one contained ethyl- α -keto- β -methyl-valerate. The animals represented by curve VII were given diet 3 (without isoleucine but with ethyl- α -keto- β -methyl-valerate) during the first period; after the time marked by the arrow, they were given diet 7 (diet 3 + 4% of DL-isoleucine). The animals represented by curve VIII received diet 4 (without isoleucine) and thereafter diet 8 (diet 4 + 4% of DL-isoleucine). Each curve denotes the mean value for 5 rats. The figures in brackets are the mean initial and final weight, respectively.

The reason for this poor utilization may be that the organism is unable to split the esters of α -keto acids. This would imply that the esterases present in the gastro-intestinal tract would only hydrolyze the keto esters to a slight extent, or not at all. As far as I am aware, no investigations have been made of this matter. Another possible explanation is that the taste and smell of the keto esters were so unpleasant that the animals refused to eat sufficient quantities of this food. It is, however, shown by the experiments recorded in Fig. 2 that this cannot have been the case. During the first period, the two groups of animals were given a diet devoid of isoleucine, with and without ethyl- α -keto- β -methyl-valerate, respectively. The mean initial weight was

Table 2. Sucrose-vitamin mixture.

	g
Sucrose	1.500
Aneurin hydrochloride	0.050
Riboflavin	0.100
Pyridoxine hydrochloride	0.050
Nicotinamide	0.050
Calcium <i>d</i> -pantothenate	0.250
<i>p</i> -Aminobenzoic acid	3.000
2-Methyl-1,4-naphthoquinone	0.020
Folic acid	0.001
Biotin	0.001

Table 3. Change in the body weight in growing rats given diets without valine, leucine and isoleucine, respectively, and diets in which these amino acids were replaced by the ethyl esters of the corresponding α -keto acids.

At the beginning of the experiments, each group comprised 10 animals. The values recorded are the means with their standard errors. The initial weight given in brackets denotes the mean for the 5 animals on which the experiments were continued more than 10 days.

Experi- mental series	No.	Diet	Initial weight	Change in body weight (g) after			Change in weight after 10 days, corresponding approx. to a con- centration of the resp. L-amino acid of
				10 days	18 days	25 days	
I	1	Valine replaced by ethyl- α -ketoiso- valerate	60 ± 2.1 (58 ± 1.9)	-8 ± 0.8	$-13 \pm 0.9^*$		0.1 %
	2	Without valine and the ester	54 ± 1.1 (56 ± 1.6)	-11 ± 0.6	$-19 \pm 2.7^{**}$		
II	3	Isoleucine replaced by ethyl- α -keto- β - methyl-valerate	55 ± 2.0 (59 ± 2.0)	-4 ± 1.4	$-11 \pm 1.0^*$	$-14 \pm 0.9^*$	0.2 %
	4	Without isoleucine and the ester	55 ± 2.5 (62 ± 1.9)	-13 ± 1.1	$-22 \pm 1.5^*$	$-26 \pm 1.7^*$	
III	5	Leucine replaced by ethyl- α -ketoiso- capronate	58 ± 1.5 (57 ± 2.0)	-5 ± 0.8	$-3 \pm 1.5^*$	$-3 \pm 1.7^*$	0.1 %
	6	Without leucine and the ester	65 ± 3.5 (64 ± 7.2)	-13 ± 0.9	$-17 \pm 1.7^*$	$-20 \pm 1.9^*$	

* After 10 days, the group comprised 5 animals only.

** 3 out of the 5 animals died between the 12th and 18th days.

about the same, i.e., 51 ± 2.6 g in the group given the ethyl ester, and 49 ± 1.9 g in the group without it. After 12 days on the respective diets, the change in body weight was -2 ± 3.0 g and -13 ± 0.7 g, respectively, the difference between the two figures being significant. This implies that part of the ester of ketoisoleucine was converted into isoleucine. When the two groups of animals were subsequently fed on the same diets as earlier, but with the addition of 4 % of DL-isoleucine, a rapid gain in weight was noted in both groups. During the 21 days from the day on which the diets were changed, the gain in weight amounted to 19 ± 3 g in the group previously on a diet with the ethyl ester of ketoisoleucine, and 27 ± 2 g in the group on the diet without this ester. The gain in weight was thus greater in the group earlier given the diet that did not contain the ester. This difference can, however, presumably be ascribed to the fact that one group of animals was more protein-depleted than the other, and not to any difference in the nutritional value of the respective diets. It has been established by earlier experiments that growth is more rapid after protein depletion. During the first 10 days after the change to diet 8, the average gain in weight was 16 g, whereas in group XII

with diet 7, it was 12 g. The normal increase in weight with the diet in question is 12 g per 10 days ⁵. This shows that the presence of the ethyl ester of ketoisoleucine has little effect on growth with a diet containing all the essential amino acids. Consequently, the experiments imply that the ethyl esters of the keto acids do not inhibit the normal synthesis of protein. The poor growth produced by the ethyl esters of the keto analogues of the amino acids is probably dependent on the inability of the organism to hydrolyze them into the α -keto acids.

SUMMARY

Experiments are made to ascertain the extent to which the ethyl esters of the α -keto analogues of valine, isoleucine and leucine are able to produce growth when the respective amino acids are lacking in the diet. It is found that these esters are unable to induce growth in young rats.

When these ethyl esters are given, a reduction is nevertheless obtained in the loss of weight occurring in the absence of the respective amino acids from the diet. This is interpreted as an indication that a small proportion (5 to 7 %) of the ethyl ester is converted into the relevant amino acid.

The explanation of the results is considered to be the inability of the organism to split these ethyl esters.

An account is given of the synthesis of ethyl- α -ketoisovalerate, ethyl- α -keto- β -methyl-valerate and ethyl- α -isocaproate.

REFERENCES

1. Wretling, K. A. J. *Acta Physiol. Scand.* **30** (1949) 97.
2. Rupe, H. and Courvoisier, C. *Helv. Chim. Acta* **6** (1923) 1061.
3. Sen, H. K. *Biochem. Z.* **143** (1923) 198.
4. Wretling, K. A. J. *Acta Physiol. Scand.* **27** (1952) 183.
5. Wretling, K. A. J. *Ibid.* **17** (1949) Suppl. 59.
6. Jones, J. H. and Foster, C. J. *Nutr.* **24** (1942) 245.

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