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Amino Acid-Activating Enzymes in Muscle

By R. J. PENNINGTON

Departments of Pathology (Chemical Pathology) and Medicine (Neurology), King's College (University of Durham) and Royal Victoria Infirmary, Newcastle upon Tyne

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The enzymic 'activation' of amino acids (Hoagland, 1955) is recognized and measured by the formation of hydroxamic acids in the presence of amino acids, adenosine triphosphate and hydroxylamine, or by the incorporation of radioactive pyrophosphate into adenosine triphosphate in the presence of amino acids. There is ample evidence (e.g. Kingdon, Webster & Davie, 1958) that activation involves the reaction of the amino acid with adenosine triphosphate to form an amino acid-adenylic acid anhydride and pyrophosphate. Magnesium appears to be essential for this reaction, which is generally considered to be the first step in the synthesis of protein from amino acids. It seems likely that separate activating enzymes for all the individual amino acids exist. Some have been purified, e.g. for methionine (Berg, 1956), tyrosine (Van de Ven, Konigsberger & Overbeek, 1958), valine (Berg & Ofengand, 1958), tryptophan (Davie, Konigsberger & Lipmann, 1956), alanine (Goldstein & Holley, 1960) and serine (Webster & Davie, 1959).

The presence of such enzymes activating various amino acids has been demonstrated in many animal and plant tissues and in micro-organisms (e.g. Nismann, Bergmann & Berg, 1957; Marcus, 1959; McCorquodale & Mueller, 1958; Webster, 1959; Lipmann, 1958; Novelli, 1958; Cole, Coote & Work, 1957). In animal tissues they have been studied largely in the 'soluble' fraction obtained after high-speed centrifuging of tissue homogenates. Recently (Reis, Coote & Work, 1959; Craddock & Simpson, 1960), however, their presence in mitochondria has been demonstrated.

No study of amino acid-activating enzymes in skeletal muscle has yet been reported. This paper

describes such experiments, carried out with mouse and human skeletal muscle. In addition, similar measurements were made with muscle from mice with hereditary muscular dystrophy, as part of a study of muscle-protein synthesis in this disease.

EXPERIMENTAL

Mice. All the mice used were of the inbred strain 129, bred from animals obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, U.S.A. This strain carries a recessive gene which produces, in the homozygote, a progressive muscular dystrophy (Michelson, Russell & Harman, 1955). The 'normal' mice of this strain may be either homozygous (double dominant) or heterozygous with respect to the gene. The animals were fed without restriction on 'breeding sow cubes' (J. N. Brown and Son, Minden Street, Newcastle upon Tyne) supplemented daily with fresh vegetables and were used when 2-3 months old. All the affected mice which were used had been frequently observed to drag their hind legs behind them.

Human muscle. Human quadriceps muscle was obtained in the course of orthopaedic operations.

Preparation of muscle 'cell-sap' fraction. The mice were killed by dislocation of the neck and the muscle from the hind legs was removed as quickly and completely as possible into a cold (0-2°) solution of 0.02M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) (obtained by dilution of a 2M-solution which had been adjusted to pH 7.4 with HCl) and 0.05M-KCl. Human muscle was placed in a similar solution immediately upon removal. Visible fat was removed and the muscle chopped finely with scissors. It was homogenized in 4-5 vol. of tris-KCl in a Potter-Elvehjem homogenizer with a Perspex pestle; a loosely fitting tube was used first with subsequent treatment in a tighter tube to break up the fibres completely. The homogenate was centrifuged for 1 hr. at 70 000 g in a Spinco model L ultra-centrifuge.

If the supernatant was used without further treatment extremely high blank values were obtained, presumably due largely to amino acids in the supernatant; the percentage exchange of pyrophosphate with adenosine triphosphate (ATP) in the absence of added amino acids was about one-half of that obtained when a complete mixture of amino acids was added. This could be decreased to about one-fifth by overnight dialysis at about 2° against tris-KCl. However, exchange in the presence of added amino acids was appreciably decreased by this treatment. McCorquodale & Mueller (1958) likewise reported that amino acid activation in rat uterus was decreased by dialysis for 20 hr. Treatment of the muscle supernatant (2 ml.) with Zeo-Karb 225 (K⁺ form; 200 mg.) decreased its exchange activity both in the absence and in the presence of added amino acids to less than one-half.

Removal of water and compounds of small molecular weight by means of Carbowax (polyethyleneglycol) enclosed in dialysis tubing was found to be more rapid than dialysis as a means of decreasing the blank values. (The use of Carbowax for concentrating protein solutions was suggested by Kohn, 1959.) A length of $\frac{1}{2}$ in. Visking tubing containing Carbowax 20 M (obtained from G. T. Gurr Ltd., 136 New Kings Rd., London, S.W. 6) was wound spirally on a glass rod and immersed in the muscle supernatant in a test tube standing in ice. After about 2 hr. the volume of liquid, which had decreased to one-fifth or less, was rediluted with tris-KCl. The whole procedure was repeated

once with fresh Carbowax. A portion of the final preparation was kept for protein determination by the biuret method (Gornall, Bardawill & David, 1949).

Hydroxamic acid formation. The tissue preparation (0.4 ml.) was incubated at 38° for 1 hr. in the following medium (total volume, 1.3 ml.): hydroxylamine, pH 7.4 (prepared according to Beinert *et al.* 1953), 0.77 M; MgCl₂, 7.5 mM; ATP, 7.5 mM; complete amino acid mixture (see below), 2 μ moles of each amino acid. Hydroxamic acid formation was measured by the procedure of Hoagland, Keller & Zamecnik (1956).

³²P-exchange studies. The tissue preparation (0.2 ml.) was incubated at 38° for 15 min. in the following medium: tris (pH 7.4 at 20°), 50 mM; MgCl₂, 10 mM; ATP (cryst. disodium salt, adjusted to pH 7.4 with KOH), 3 mM; Na₄³²P₂O₇, 3 mM; NaF, 25 mM; amino acids (1 μ mole of each, neutralized where necessary), as indicated. The total volume was 0.5 ml. The concentrations given were those after the addition of the tissue preparation. The complete amino acid mixture (pH 7.4) contained glycine and the following L-amino acids: alanine, arginine, aspartic acid, cysteine, cystine, glutamic acid, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine. The tyrosine and cystine did not completely dissolve.

Tubes were always incubated in duplicate. After incubation 2 ml. of 6.25% trichloroacetic acid was added and the precipitate removed by centrifuging. The ATP was separ-

Table 1. *Amino acid activation (³²P exchange) by cell-sap from normal mouse muscle*

The incorporation of pyrophosphate into ATP was measured in the presence of the amino acids indicated. For details, see text. Each numbered experiment was carried out with pooled tissue from two mice.

Amino acid or peptide	Percentage incorporation of ³² P into ATP/mg. of protein								
	1	2	3	4	5	6	7	8	9
None	1.57	2.72	2.85	2.62	2.89	3.02	2.74	2.61	2.46
Complete mixture	11.4	18.3	15.6	20.9	16.3	19.9	15.7	16.8	9.85
L-Alanine	—	4.34	—	—	3.42	—	3.44	—	—
L-Arginine	—	—	2.75	2.13	—	—	—	—	2.31
L-Aspartic acid	—	—	2.86	3.18	—	—	—	—	—
L-Cysteine	—	4.37	—	—	—	—	4.36	—	—
L-Glutamic acid	—	—	2.92	2.27	—	—	—	2.28	—
Glycine	1.53	—	—	—	3.22	—	—	2.63	—
L-Histidine	—	—	3.35	3.03	—	3.52	—	—	—
L-Hydroxyproline	—	—	2.90	—	—	—	2.78	—	2.41
L-Isoleucine	2.12	—	—	—	5.92	—	—	—	—
L-Leucine	3.91	—	—	8.52	—	9.10	8.00	—	—
L-Lysine	—	—	3.72	—	—	—	3.75	—	—
L-Methionine	—	3.02	—	—	3.67	—	—	2.73	—
L-Phenylalanine	—	2.66	—	—	—	—	2.66	—	2.40
L-Proline	—	—	4.56	—	5.19	—	—	—	—
L-Serine	—	3.32	—	—	4.44	—	—	—	—
L-Threonine	2.74	—	—	—	4.07	—	—	—	—
L-Tryptophan	—	11.0	—	8.98	—	8.67	—	4.43	—
L-Tyrosine	—	—	3.78	—	—	—	3.96	—	—
L-Valine	3.42	—	—	—	6.72	—	—	—	—
L-Carnosine	—	—	3.72	2.88	—	—	—	—	—
Glutathione	—	—	—	—	—	—	—	—	3.27
l-Methyl-DL-histidine	—	—	—	—	—	—	—	—	2.48
D-Tryptophan	—	—	—	—	—	3.02	2.54	—	—
L- and *D-Tryptophan	—	—	—	—	—	5.61	—	3.12	—
L-Leucine + *D-tryptophan	—	—	—	—	—	—	8.00	—	—
Protein added (mg.) ...	2.12	1.84	2.08	1.56	1.74	1.89	1.40	1.08	1.36

* 4 μ moles of D-tryptophan were added.

ated with charcoal by a procedure based on that of Crane & Lipmann (1953). A quantity of charcoal (100 mg.) was stirred with the trichloroacetic acid supernatant and separated by centrifuging. It was washed twice with about 5 ml. of $\text{mm-Na}_4\text{P}_2\text{O}_7$, and once with the same quantity of water, then heated with 1 ml. of N-HCl for 15 min. at 100° . The mixture was filtered and the charcoal well washed with water. Portions of the combined filtrates were evaporated on polythene planchets and the radioactivity was measured with an end-window counter; the standard deviation of the counts was never more than 2%. Inorganic phosphate in the filtrate was determined by the method of Fiske & Subbarow (1925). Tubes without ATP were included in each experiment. The corresponding charcoal eluates contained a small amount of inorganic phosphate (approx. 10% of that obtained from tubes with ATP). After subtraction of this blank value the inorganic phosphate values were used to correct the counts for the loss of ATP (about 20%) during the separation. Zero-time controls showed that there was no hydrolysis of ATP during the incubations. The percentage of the exchange possible was calculated as

$$100 \times \frac{\text{counts/min. in ATP}}{\frac{1}{2} (\text{initial counts/min. in pyrophosphate})}$$

Materials. The amino acids were all commercial preparations. None of those used, when examined by two-dimensional paper chromatography, showed any additional spots. The solvents used for chromatography were butanol-pyridine-water (1:1:1, by vol.) and phenol-ammonia [500 g. of phenol was mixed with 125 ml. of water; after standing overnight, 1 ml. of aq. conc. NH_3 soln./200 ml. was added (Smith, 1958)]. The chromatography was carried out for approx. 18 hr. on Whatman no. 1 paper.

Radioactive sodium pyrophosphate was obtained from The Radiochemical Centre, Amersham, Bucks.

Activated charcoal (British Drug Houses Ltd.) was washed several times with hot (90°) HCl (1 vol. of HCl , sp.gr. 1.18:5 vol. of water) and then with distilled water until the washings were neutral and dried *in vacuo* over CaCl_2 .

RESULTS AND DISCUSSION

In the presence of the complete mixture of amino acids the muscle preparation formed $0.050 \mu\text{mole}$ of hydroxamic acid/mg. of protein/hr.; in the absence of added amino acids $0.017 \mu\text{mole}$ was formed. A corresponding preparation from mouse liver formed $0.095 \mu\text{mole}$ /mg. of protein/hr. in the presence of amino acids. In view of the greater sensitivity of the pyrophosphate-exchange method all studies with individual amino acids were carried out with it. Table 1 shows the results obtained with normal mouse muscle. Most of the amino acids increased the exchange. Although there were some differences between the relative exchange rates in different experiments, there is a quite consistent pattern of activation. Leucine and tryptophan were most strongly activated. Arginine, hydroxyproline, glutamic acid and phenylalanine consistently failed to stimulate the ^{32}P exchange. The lack of effect of an added amino acid could, of course, be due to inactivation of the

appropriate enzyme or to the retention of an adequate quantity of amino acid by the muscle preparation. The dipeptide carnosine, a normal constituent of muscle of unknown function, showed some activity. Methylhistidine, which is excreted in the urine in muscular dystrophy induced by vitamin E deficiency (Fink, Williams & Fink, 1959), had no effect. D-Tryptophan was apparently not activated and inhibited the activation of the L-isomer.

Doubling the quantity of muscle, in the presence of the amino acid mixture, doubled the incorporation of ^{32}P . Incubation for 30 min. instead of the usual 15 min. almost doubled the incorporation. A cell-sap preparation from mouse liver, prepared in a similar manner, produced 32% exchange of labelled pyrophosphate/mg. of protein/15 min.

Table 2. *Effect of concentration of L-leucine upon ^{32}P exchange*

See text for details.

L-Leucine added ($\mu\text{moles}/0.5 \text{ ml.}$)	Percentage incorporation of ^{32}P into ATP/mg. of protein	
	1	2
None	2.26	2.46
0.01	3.05	—
0.10	4.18	—
1.00	5.10	5.78
5.00	—	5.79

Table 3. *Amino acid activation by cell-sap from human skeletal muscle*

The incorporation of pyrophosphate into ATP was measured in the presence of the amino acids indicated. For details, see text.

Amino acid	Percentage incorporation of ^{32}P into ATP/mg. of protein	
	1	2
None	2.84	2.12
Complete mixture	20.7	9.83
L-Alanine	4.85	—
L-Arginine	—	2.04
L-Aspartic acid	—	2.21
L-Cysteine	—	3.55
L-Glutamic acid	2.85	—
Glycine	3.42	2.64
L-Histidine	4.32	—
L-Hydroxyproline	—	1.94
L-Isoleucine	—	2.66
L-Leucine	3.45	3.67
L-Lysine	7.05	—
L-Methionine	—	2.48
L-Phenylalanine	2.86	—
L-Proline	6.17	—
L-Serine	—	2.22
L-Threonine	4.69	—
L-Tryptophan	11.89	—
L-Tyrosine	—	2.36
L-Valine	4.40	—
Protein added (mg.) ...	1.39	1.72

Table 4. *Amino acid activation by cell-sap from dystrophic mouse muscle*

The incorporation of pyrophosphate into ATP was measured in the presence of the amino acids indicated. For details, see text.

Amino acid or peptide	Percentage incorporation of ³² P into ATP/mg. of protein			
	1	2	3	4
None	2.63	3.01	3.12	1.93
Complete mixture	14.1	8.73	11.0	7.30
L-Alanine	—	3.41	4.01	2.26
L-Arginine	—	—	3.09	—
L-Aspartic acid	—	—	—	2.11
L-Cysteine	—	—	4.62	—
L-Glutamic acid	—	—	—	2.00
Glycine	—	—	3.14	—
L-Histidine	—	3.36	3.61	2.15
L-Hydroxyproline	—	—	—	1.92
L-Isoleucine	—	3.60	4.58	—
L-Leucine	7.75	—	—	—
L-Lysine	—	3.24	—	2.09
L-Methionine	—	—	3.42	—
L-Phenylalanine	—	—	3.20	—
L-Proline	2.97	—	3.64	2.09
L-Serine	—	—	3.54	2.12
L-Threonine	—	3.79	4.09	—
L-Tryptophan	5.03	—	—	—
L-Tyrosine	—	—	—	2.27
L-Valine	4.57	—	—	—
L-Carnosine	—	—	—	2.12
Glutathione	—	—	—	2.40
Protein added (mg.) ...	0.70	1.18	0.86	0.75

The effect of the concentration of an amino acid (L-leucine) upon the incorporation is shown in Table 2. The amino acid increased the incorporation appreciably at a concentration of 0.02 mM.

Table 3 shows similar experiments with human muscle, which displayed a broadly similar pattern of activation.

Table 4 records experiments with tissue from dystrophic mice. In spite of the considerable variation between experiments the percentage incorporation/mg. of protein, in the presence of the amino acid mixture, was significantly (P 0.01–0.02) lower for the dystrophic mice (mean values 16.1 and 10.2 respectively for normal and dystrophic mice). The dystrophic muscle appears to be capable of activating all the amino acids which are activated by the normal muscle.

SUMMARY

1. The 'cell-sap' fraction of mouse skeletal muscle incorporated labelled pyrophosphate into adenosine triphosphate in the presence of L-amino acids. Leucine and tryptophan were most effective. Most L-amino acids were active.

2. D-Tryptophan (8 mM) inhibited incorporation in the presence of L-tryptophan (2 mM) by approximately one-third.

3. The relative activities of the individual amino acids were roughly the same when a similar preparation from human skeletal muscle was used.

4. Muscle cell-sap from mice with hereditary muscular dystrophy showed on the average less incorporation/mg. of protein, although there was large individual variation. The mean percentage incorporation/mg. of protein/15 min. was 16.1 and 10.2 respectively for normal and dystrophic mice.

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