Some Characteristics of the Enzymic Carboxyl Activation of Methionine

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Hoagland (1955) reported the presence in rat liver of enzymes which catalyse the formation of adenylates from amino acids and adenosine triphosphate (ATP), and suggested the following scheme (PP represents pyrophosphate) for the reaction:

The binding of the carboxyl group of the amino acids to the adenosine monophosphate (AMP) is considered as an activation of the carboxyl group. This activation was confirmed by the fact that in the presence of hydroxylamine it was possible to demonstrate the presence of the corresponding hydroxamates of the amino acids, according to the reaction:

Later, Hoagland, Keller & Zamecnik (1956) extended those observations and tested the specificity of different enzyme preparations towards the various amino acids. They were able to purify, by ammonium sulphate fractionation of the liver extract, an enzyme which was specific for the activation of the carboxyl group of methionine. Davie, Konigsberger & Lipmann (1956), starting with pancreas, obtained an enzyme preparation which was specific for tryptophan. Later they studied the specificity of this enzyme towards tryptophan analogues with a view to determining the role of these enzymes in protein synthesis (Sharon & Lipmann, 1957).

Although the role of peptides in protein synthesis is not yet clear it is of interest to determine whether an enzyme preparation which is specific for the activation of an amino acid activates the amino acid when it is in a peptide linkage. The specificity of the methionine-activating enzyme towards methionine peptides has therefore been studied. The action of various metabolic inhibitors on the enzyme activity has also been studied to determine whether the inhibition of protein synthesis by such compounds can be related to the

inhibition of these enzymes. A preliminary report of some aspects of these results has been published (Rendi, 1956).

MATERIALS AND METHODS

Enzyme preparation. Wistar albino rats weighing 150–200 g. were used. The animals were killed by bleeding and the liver was removed as rapidly as possible. The methionine-activating enzyme was extracted with 0.05 M-KCl, and the extract was fractionated with ammonium sulphate between 35 and 45% of saturation as described by Hoagland *et al.* (1956).

Fractionation of the liver. Rat liver was homogenized with 2 vol. of 0.25 M-sucrose in a Teflon (polytetrafluoroethylene) Potter-Elvehjem-type homogenizer and the suspension was centrifuged at 18 000 g in an International refrigerated centrifuge for 1 hr. at +1°. The pellet was resuspended and homogenized with 1 vol. of 0.25 M-sucrose and centrifuged in a similar manner. The combined supernatants were dialyzed for 20 hr. at +2° against 0.05 M-KCl, and the dialyzed supernatant was fractionated with ammonium sulphate. The pellet, containing nuclei, mitochondria and microsomes, was homogenized with 3 vol. of 0.05 M-KCl, and centrifuged for 1 hr. at 18 000 g, and the supernatant treated with ammonium sulphate.

Measurement of activation. The reaction mixture contained, in 1 ml. of 0.1 M-2-amino-2-hydroxymethylpropane-1,3-diol buffer (pH 7.6), 1.2 m-mole of hydroxylamine (free base), 10 μmoles of Mg²⁺ ion, 10 μmoles of adenosine triphosphate (ATP) (Sigma Chemical Co., St Louis, Mo., U.S.A.), brought to pH 7.6 with NaOH, and an appropriate amount of the enzyme [between 1.5 and 3 mg. of protein determined by the method of Gornall, Bardawill & David (1949) in the final 1 ml.] and the different substances to be tested. The mixture was incubated at 37° for 2 hr., and the quantity of hydroxamate was determined by the method of Hoagland *et al.* (1956). This method depends on the formation of a red colour when FeCl₃ in acid solution is added to hydroxamates. In a typical experiment the amount of hydroxamate formed in the presence of 12 μmoles of methionine is between 1.2 and 2.5 μmoles. Because of this great variation in the amount of hydroxamate formed, the data are reported as a percentage of the amount formed in the presence of methionine.

Hydroxylamine was prepared from hydroxylamine hydrochloride dissolved in methanol by neutralizing with saturated NaOH in methanol. The precipitated NaCl was filtered off, and the free base was precipitated by adding to the filtrate an equal amount of ether. The precipitate was washed with methanol-ether mixture and ether.

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Table 1. *Intracellular localization of the methionine-activating enzyme*

Results are expressed as a percentage of the colour intensity with methionine as substrate with the enzyme preparation from the soluble fraction of the cytoplasm. The particulate fraction contained nuclei, mitochondria and microsomes. The three experiments were carried out with different tissue preparations, and data reported are the mean value of the experiment $\pm$ the standard deviation.

No. of experiments	Intracellular fraction	Hydroxamate formed	
		Without methionine	With 12 μ moles of methionine
3	A 0.05M-KCl extract of the particulate fraction	10 $\pm$ 2	12 $\pm$ 2
3	Soluble fraction of cytoplasm	30 $\pm$ 3	100

Table 2. *Enzymic formation of methionine hydroxamate in the presence of nucleic acids*

Results are expressed as a percentage of the colour intensity with methionine as substrate. In each experiment 0.5 mg. of nucleic acid in the final 1 ml. volume was added. The data represent the mean value $\pm$ the standard deviation.

No. of experiments	Substances added	Hydroxamate formed	
		Without methionine	With 12 μ moles of methionine
9	None	30 $\pm$ 2	100
5	RNA	28 $\pm$ 3	68 $\pm$ 4
5	RNA hydrolysate	35 $\pm$ 3	82 $\pm$ 5
3	DNA	34 $\pm$ 2	99 $\pm$ 3

Fig. 1. Enzymic formation of methionine hydroxamate in the presence of an enzymic hydrolysate of RNA with different concentrations of hydroxylamine. The hydroxamate formed is expressed as a percentage of the colour intensity observed with methionine alone at the highest hydroxylamine concentration. O, Without RNA hydrolysate; ●, with RNA hydrolysate (0.5 mg. in the final 1 ml. volume).

Ribonucleic acid hydrolysate. A portion (200 mg.) of thymus ribonucleic acid (RNA) (Bayer, Leverkusen, Germany) was dissolved in 0.1M-2-amino-2-hydroxy-methylpropane-1:3-diol buffer (pH 6.6) with 50 μ g. of crystalline ribonuclease (Sigma Chemical Co.), and the solution was dialysed for 24 hr. at room temperature. The ribose concentration in the diffusate was determined colorimetrically (Dische & Schwartz, 1937) and the solution was diluted to the required concentration.

Other substances. All amino acids and peptides were DL. Protamine was a salmine sulphate preparation obtained from British Drug Houses Ltd., and yeast deoxyribonucleic acid was obtained from Bayer. The metabolic inhibitors were obtained from Merck, Darmstadt, Germany.

RESULTS

Intracellular localization of the activating enzyme. The fractions purified from the particulate fractions or from the soluble cytoplasm of rat liver were brought to equal protein concentrations (between 2.8 and 3.0 mg. of protein in the final volume of 1 ml.) and their activating capacity was tested with methionine.

Table 1 shows that only the enzyme preparation from the soluble fraction of cytoplasm catalysed the formation of hydroxamate in the presence of methionine, whereas the preparation from the particulate fractions of liver was inactive.

Action of nucleic acids on formation of hydroxamate. Hoagland & Zamecnik (1957) suggested that in a cell-free system the amino acids after their activation become associated with RNA molecules. Therefore the action of nucleic acids, and of an enzymic hydrolysate of RNA, on the enzymic formation of methionine hydroxamate was studied. In *Staphylococcus* (according to Gale, 1955) the enzymic hydrolysate of RNA stimulates protein synthesis, as does the undegraded RNA.

Table 2 shows that the presence of RNA or of RNA hydrolysate inhibits hydroxamate formation, whereas DNA is ineffective. The inhibitory effect of the RNA hydrolysate is smaller than that observed with the unhydrolysed RNA. The curves presented in Fig. 1 show that the hydroxylamine concentration does not influence the inhibition exerted by the RNA hydrolysate, which is always approximately 20 %.

Activation of peptides. As shown in Table 3, two different peptides containing methionine with its carboxyl group in a terminal position are activated by the purified enzyme, the hydroxamate formation in the presence of alanylmethionine being lower than that of the others. The determinations of hydroxamate formation with different quantities of substrates (Fig. 2) show that the rate

of formation of hydroxamate is less for alanyl-methionine at all concentrations and is 60 % of that formed in the presence of methionine. Although experimental data on the intensity of the colour reaction between FeCl_3 and peptide hydroxamates have not been reported, it is known that the colour intensities in the FeCl_3 reaction produced by various amino acid hydroxamates differ up to 20 % (Schweet, 1955). The value obtained for alanyl-methionine hydroxamate formation is so much below that of methionine that it can hardly be accounted for by a difference in the production of colour intensity.

Effects of inhibitors of protein synthesis. Among the different inhibitors of protein synthesis three groups of substances were studied: (i) amino acid analogues (ethionine 12 mM); (ii) the substances which interfere with nucleic acid metabolism (ribonuclease and protamine); (iii) inhibitors of oxidative phosphorylation and glycolysis (NaN_3 , 2:4-dinitrophenol, NaF, all at 25 mM).

Table 3. *Hydroxamate formed by the purified enzyme with different substrates*

Results are expressed as a percentage of the colour intensity with methionine as substrate. In each experiment 12 μmoles of substrate in the final 1 ml. volume were added. The data are reported as mean value $\pm$ standard deviation.

No. of experiments	Substrate	Hydroxamate formed
10	None	30 $\pm$ 2
12	Methionine	100
6	Glycylmethionine	100 $\pm$ 4
6	Alanylmethionine	60 $\pm$ 3

Fig. 2. Effect of different concentrations of substrates on the enzymic formation of hydroxamates. The hydroxamate formed is expressed as a percentage of the colour intensity observed for the higher methionine concentration. ○—○, Methionine; ●—●, glycylmethionine; — — —, alanylmethionine.

Fig. 3. Enzymic activation of methionine in the presence of ethionine (12 μmoles of each amino acid in the final 1 ml. volume). The hydroxamate formed is expressed as a percentage of the colour intensity observed for methionine alone at the end of the experiment. ○, Without ethionine; ●, with ethionine.

Table 4. *Enzymic formation of methionine hydroxamate in the presence of ribonuclease and protamine*

Results are expressed as a percentage of the colour intensity with methionine as substrate. In each experiment 50 $\mu\text{g.}$ of ribonuclease and 0.5 mg. of protamine in the final 1 ml. volume were added. The data are reported as mean value $\pm$ standard deviation.

No. of experiments	Substance added	Hydroxamate formed	
		Without methionine	With 12 μmoles of methionine
8	None	30 $\pm$ 2	100
5	Ribonuclease	38 $\pm$ 3	107 $\pm$ 4
4	Protamine	42 $\pm$ 4	110 $\pm$ 5

Table 5. *Enzymic formation of methionine hydroxamate in the presence of inhibitors of oxidative phosphorylation and glycolysis*

Results are expressed as a percentage of the colour intensity with methionine as substrate. In each experiment 12 μmoles of methionine and 25 μmoles of the metabolic inhibitors were added. The data are reported as mean value $\pm$ standard deviation.

No. of experiments	Substance added	Hydroxamate formed
8	Methionine	100
4	Methionine and NaF	99 $\pm$ 4
4	Methionine and NaN_3	100 $\pm$ 5
4	Methionine and dinitrophenol	101 $\pm$ 4

The failure of ethionine to inhibit the formation of methionine hydroxamate during incubation for 2 hr. is shown in Fig. 3. It has also been observed that ethionine is not activated by the methionine-activating enzyme and also that ethionine does not inhibit the formation of hydroxamates of methionine peptides.

The action of ribonuclease and protamine upon methionine hydroxamate formation is summarized in Table 4. It can be seen that in the presence of these compounds there is an increase in hydroxamate formation, but that such an augmentation is also found in the control experiments with ribonuclease or protamine but no amino acids present. Therefore it might be assumed that such compounds act as substrates for the enzyme preparations and in any case do not inhibit hydroxamate formation.

Table 5 shows the action of inhibitors of protein synthesis, which interfere with oxidative phosphorylation and glycolysis. NaF, NaN₃ and dinitrophenol did not affect the hydroxamate formation. It was not possible to test the action of α , α '-dipyridyl and monoiodoacetate, because the first compound reacts with FeCl₃ and with the second a non-enzymic reaction with hydroxylamine occurs which yields a compound that reacts with the ferric chloride reagent.

DISCUSSION

The results presented here show that no methionine-activating enzyme can be extracted from the particulate fraction of rat liver with 0.05M-KCl. This observation is consistent with the data reported by Zamecnik & Keller (1954) and those reported by Hoagland *et al.* (1956), who found that soluble cytoplasm is necessary for incorporation of amino acids into microsomal proteins, and that the soluble fraction of cytoplasm can be replaced in this case by a protein fraction of the cytoplasm containing activating enzymes.

The idea that RNA plays a role in protein synthesis is now widely accepted. The hypothesis of Zamecnik, Keller, Hoagland & Littlefield (1956) was recently confirmed by Hoagland & Zamecnik (1957) by showing that radioactive amino acids are transferred after their activation to a soluble RNA fraction. The present results, showing that RNA inhibits the formation of hydroxamate, can also be taken as an indication that RNA participates in a step of protein synthesis related to activation of amino acids, although the RNA used, being extracted from yeast, is not specific for liver-activating enzymes, and more experiments should be made with RNA prepared from liver.

It was previously shown (Rendi, 1955) that the hydroxamate formation in the presence of an amino

acid mixture catalysed by the soluble fraction of rat liver described by Hoagland (1955) was the same if glycine in the mixture was replaced by diglycine or triglycine, and it was suggested that the carboxyl group of diglycine and triglycine, like that of glycine, was activated by liver. In the present paper it is shown that peptides containing methionine with its carboxyl group in the terminal position are also activated by the enzyme preparation which activates the methionine carboxyl group only. These results differ from those reported by Davie *et al.* (1956), working with the tryptophan-activating enzyme. This enzyme did not activate either *N*-acetyltryptophan or glycyltryptophan, in contrast with glycylmethionine and the methionine-activating enzyme. It should, however, be mentioned that the tryptophan-activating enzyme is much purer than the enzyme preparation that was used in this investigation (Hoagland *et al.* 1956), and the possibility that this preparation also contains the enzymes specific for the activation of methionine peptides cannot be excluded. It is, however, unlikely that a different enzyme exists for the specific activation of each peptide; it is more probable that not all peptides can be activated. The activation of peptides reported here supports the possibility that such compounds could be incorporated into proteins without previous hydrolysis to free amino acids.

The findings of a lack in inhibition of the methionine-activating enzyme by sodium fluoride, sodium azide and dinitrophenol is in agreement with the supposition that such compounds inhibit amino acid incorporation into proteins by interfering with the mechanisms which provide the energy for the formation of peptide bonds.

The observation reported above, that ribonuclease and protamine do not inhibit the activity of the methionine-activating enzyme, supports the hypothesis that RNA has no direct action on the initial step of protein synthesis. A finding which points in the same direction is that of Davie *et al.* (1956), who found that their purified tryptophan-activating enzyme contained nucleotides, chiefly guanosine monophosphate, but that it did not lose any of its activity when deprived of this.

Another characteristic which distinguishes the methionine- from the tryptophan-activating enzyme is its behaviour in the presence of amino acid analogues. Davie *et al.* (1956) and later Sharon & Lipmann (1957) observed that the tryptophan-activating enzyme can also activate 2-azatryptophan, 7-azatryptophan and 5-fluorotryptophan but not 5- or 6-methyltryptophan or 2-aza-6-methyltryptophan. They suggest that this enzyme activates only those analogues which are incorporated into the proteins (i.e. the first three). In contrast, in the present study, ethionine, the characteristic

analogue of methionine, did not form the corresponding hydroxamate in the presence of the methionine-activating enzyme and did not inhibit methionine activation. The inability of the methionine-activating enzyme to use ethionine as a substrate is in contrast with the observed incorporation of radioactive ethionine into liver proteins (Levine & Tarver, 1951) and into protein in Ehrlich ascites cells (Rabinovitz, Olson & Greenberg, 1957). However, Rabinovitz *et al.* (1957) observed that less ethionine is incorporated into proteins than methionine, and this difference could account for the inability to demonstrate ethionine activation in isolated enzyme preparations.

SUMMARY

1. Rat-liver homogenate prepared in 0.25M-sucrose was separated into particulate and supernatant fractions. The presence of an enzyme activating the carboxyl group of methionine could be demonstrated in the supernatant, but no such activity was found in a 0.05M-potassium chloride extract of the particulate fraction.

2. The enzyme was inhibited by yeast ribonucleic acid, and by an enzymic hydrolysate of ribonucleic acid, by 20–30%, but not by thymus deoxyribonucleic acid.

3. Glycylmethionine and alanylmethionine were activated by the enzyme preparation, but alanylmethionine activation was lower than that of methionine or glycylmethionine.

4. Inhibitors of protein synthesis (12 mM-ethionine, ribonuclease, protamine, 25 mM-sodium fluoride, 25 mM-sodium azide, 25 mM-dinitrophenol) failed to inhibit the enzyme activity.

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Studies on Plant Flavokinase

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As riboflavin is a component of several enzymes concerned with respiration, cells must be capable of synthesizing the active forms of the vitamin, namely both flavin mononucleotide and flavin-adenine dinucleotide. The earlier observations (Hubner & Verzar, 1939; Pulner & Verzar, 1939), that phosphorylation of riboflavin could take place by means of inorganic phosphate in preparations of intestinal mucosa, seemed unlikely in view of the energetics of the reaction. A conclusive demonstration of the phosphorylation of riboflavin by adenosine triphosphate was made by Kearney & England (1951), using a purified enzyme preparation from yeast. The only reports in the literature pertain to its isolation and purification from different yeasts (Kearney & England, 1951; Giri &

Krishnaswamy, 1956). Though the occurrence of riboflavin in plants is well known, little is known about the mechanism of synthesis of flavin nucleotides in plants. It was observed that synthesis of flavin mononucleotide took place in plant extracts when they were incubated under the same conditions as those used with yeasts (Giri, Krishnaswamy & Appaji Rao, 1957). The properties of this plant enzyme have been investigated and are described in this paper.

MATERIALS AND METHODS

Substrates and reagents. Riboflavin and flavin mononucleotide (FMN) were products of Hoffman La-Roche and Nutrition Biochemicals Corp. respectively. Adenosine