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Studies on the protein synthesis in poisoning.

III. Labeling of pH-5 enzyme with C14-glycine and the inhibition by para chloromercuribenzoate*

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Abstract

The labeling of C14-glycine to pH-5 enzyme, PP32-ATP exchange by pH-5 enzyme, and the inhibitory action of p-chloromercuribenzoate were examined. 1) The labeling of C14-glycine to pH-5 enzyme is inhibited by 10^{-4} mole of PCMB, and this inhibitory effect is reduced by addition of cysteine having 20 mole equivalent of PCMB. 2) The PP32-ATP exchange reaction is also reduced by addition of PCMB and inhibitory effect of PCMB is reduced by the addition of cysteine having 20 mole equivalent of PCMB. 3) These results show that the inhibition of the organic mercury compounds on the protein synthesis is responsible for attack the SH enzyme which catalyzes some amino-acid activation, and consequently influences activated amino-acid transfer reaction. 4) Paper electrophoretic pattern of pH-5 enzyme shows numerous peaks, each having the mobility between α 2-globulin and γ -globulin.

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STUDIES ON THE PROTEIN SYNTHESIS IN POISONING

III. LABELING OF PH-5 ENZYME WITH C¹⁴-GLYCINE AND THE INHIBITION BY PARA CHLORO- MERCURIBENZOATE

Masana OGATA

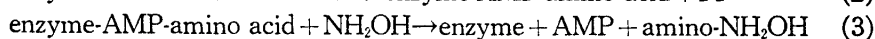
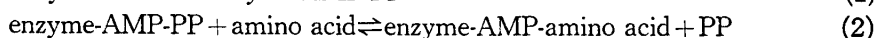
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Since the discovery of amino acid activation by an enzyme fraction from a soluble and none particulate liver fraction, designated as the pH-5 enzyme by HOAGLAND *et al.*¹, many evidences²⁻⁸ indicating amino acid activation is the first step in protein biosynthesis, have been accumulated.

The pH-5 enzyme catalyzes the exchange of labeled pyrophosphate with the terminal phosphate of ATP, the α -amino hydroxamic acid formation in the presence of hydroxamine and amino acids, and thereafter the transfer of activated amino acids on the special RNA^{2,6} contained the supernatant fraction, designated 5 RNA or pH-5 RNA. The amino acids thus bound to pH-5 RNA are the precursor of protein to be synthesized in microsomes.

For the mechanism of the amino acid activation by pH-5 enzyme, HOAGLAND and ZAMECNIK² proposed the following equations.



On the other hand, in the study on the tryptophan activating enzyme, DAVIE *et al.*⁹ found that SH group is essential for the activation of several amino acids. Recently, FRASTER *et al.*⁸, SCHWEET, *et al.*¹⁰ and OGATA *et al.*¹¹, reported that the PP³²-ATP exchange occurring with leucine is also inhibited by PCMB.

As the results of inhibition of PCMB on the activity of pH-5 enzyme, estimated in PP³²-ATP exchange reaction, the amount of activated amino acids is decreased and therefore that of C¹⁴-glycine combined with pH-5 enzyme will be naturally decreased.

Studies on the inhibitory rates of the above-two steps will clarify the relation with one another, and will present limiting factor of protein synthesis in the case of mercury poisoning.

The experiment was carried out in order to find out the effect of PCMB

on the combination of the pH-5 enzyme with C^{14} -glycine. In addition, the effect of PCMB on leucine activation by the liver pH-5 enzyme was investigated.

MATERIALS AND METHODS

The liver of albumino rats served as material for the preparation of the pH-5 enzyme by the method of HOAGLAND,² i. e. the liver was homogenized in a POTTER ELVEHJEM type homogenizer with 0.05 moles of KCl solution. After centrifugating the homogenate at 105,000 g for 30 minutes, the supernatant solution was adjusted to pH 5.1 by dropwise addition of 0.1 mole of acetic acid with constant stirring. The precipitate was dissolved into 0.1 mole of Tris buffer, pH 7.8 and used in this experiment. All operations were performed at $0^{\circ}\text{C}\sim 2^{\circ}\text{C}$ in a cold room.

For determining the pH-5 enzyme activity by PP^{32} -ATP exchange reaction, the pH-5 enzyme was incubated with PP^{32} in the reaction mixture with or without PCMB, and the radioactivity of ATP was determined. The basic incubation mixture was consisted of 0.5 moles of leucine, 4.0 millimols of ATP, 5.0 millimols of MgCl_2 , 3.3 millimols of PP^{32} , 0.1 mole of inhibitor, and the pH-5 enzyme solution (2.0 mg protein in 0.5 ml of 0.1 mole Tris buffer), totalling 1.0 ml in volume. The incubation took place at 37°C for 10 minutes. After the incubation the reaction was stopped with cold trichloroacetic acid TCA adjusted 10 per cent of final concentration. The precipitated protein was removed and ATP was adsorbed on activated charcoal as described by CRANE and LIPMANN¹². Charcoal was washed 3 times with water, added with 2 N HCl, and boiled for 15 minutes at 100°C . The supernatant obtained by centrifugation was poured into a dish and after drying, the radioactivity was counted in G. M. counter.

For determining combination of C^{14} -glycine to pH-5 enzyme, pH-5 enzyme fraction was incubated with C^{14} -glycine in the incubation mixture with or without PCMB. The incubation mixture was composed of 4.0 millimols of MgCl_2 , 4.0 millimols of ATP, 0.1 mole of inhibitor, and the pH-5 enzyme solution (20 mg of protein in 0.65 ml of 0.1 mole of Tris buffer), totalling 1.3 ml in volume. After incubation the pH-5 enzyme was precipitated by 2 volumes of 10 per cent TCA, and the precipitate was washed twice with ice cold 5 per cent TCA, then with 95 per cent ethanol, acetone-ether (3 : 1), and finally with ether. The washed precipitate was poured into a dish, and after drying radioactivity was counted in the gas flow G. M. counter.

ATP used in this experiment was the crystalline disodium salt from Pabst Laboratory, C^{14} -2-glycine was purchased from the Radioactive Chemical Center, Amersham, England, and its specific activity was 4.0 mc/mM.

Observation on pH-5 enzyme fraction was carried out by paper electrophoresis. This was conducted by the technics of GRASSMAN and HANNIG¹³. As materials

for electrophoresis, the supernatant solution obtained by centrifugation with 100,500 g was precipitated by acetic acid at pH 5.0. The precipitates thus obtained were dissolved in veronal acetate buffer and used for electrophoresis. The Holt's buffer¹⁴ (pH 8.6, μ 0.045) consisted of sodium veronal, sodium acetate, and acetic acid, was used for electrophoresis. Migration time was 8 hours. After electrophoresis the mixed solution of bromophenol blue, HgCl_2 , and acetic acid was employed to treat paper strips. After washing 3 times with 2.0 per cent acetic acid and drying, the paper strips were made translucent with liquid paraffine and densitometric readings were taken.

RESULTS

The PP^{32} -ATP exchange reaction catalysed by pH-5 enzyme in the presence of leucine and inhibitory action of PCMB and other mercuric compounds on it, were investigated. The percentage inhibition is 23.7 per cent in the presence of 10^{-4} mole PCMB and by addition of cysteine having 20 mole equivalent of PCMB 15 minutes after incubation, it is reduced to 15.8 per cent as shown in Table 1. From the above facts it is concluded that PCMB inhibits pH-5 enzyme activity by reacting with SH groups of the enzyme. The exchange reaction is also inhibited by merthiolate and mercuric acetate. The percentage inhibition of 10^{-4} mole merthiolate is 16.8 per cent and that of mercuric acetate 21.4 per cent.

Table 1. Effect of PCMB, mercuric acetate, and merthiolate on pyrophosphate exchange by pH-5 enzymes.

	C. P. M.	Per cent inhibition
Complete system *	4223	
PCMB	3383	23.7
PCMB + cysteine	3564	15.8
Merthiolate	3528	16.8
Mercuric acetate	3333	21.4

The experimental condition: Final concentration of PCMB, mercuric acetate and merthiolate are 10^{-4} mole respectively.

* Average of the two experimental results.

The inhibitory effect of PCMB on the labeling of the pH-5 enzyme with C^{14} -glycine has been clearly demonstrated (Table. 2). The percentage inhibition is 31.8 per cent in the presence of 10^{-4} mole and this inhibition is reduced down to 24.8 per cent by 20 mole equivalent cysteine added 15 minutes after incubation. This shows that the labeling of pH-5 enzyme with C^{14} -glycine is also minimized by PCMB as the results of reducing activated amino acid by inhibitory effect of PCMB on the pH-5 enzyme activity, estimated by the PP^{32} -ATP exchange reaction of the enzyme.

Table 2. Effect of PCMB on the binding of C^{14} -2-glycine to the pH-5 enzymes.

	D. W. *	C. P. M.	CPM/DW	% Combin.	% Inhibition
Complete system	24.0	2302	342.5	100.0	
PCMB	25.0	1570	233.6	68.2	31.8
PCMB+cysteine	24.6	1730	257.4	75.2	24.8

The experimental condition: Final concentration of PCMB is 10^{-4} mole.

* Dry weight (mg).

Observation of pH-5 enzyme by paper electrophoresis has proven that such numerous peaks, each having the mobility between serum α_2 -globulin and γ -globulin are observable as shown in Fig. 1. These results indicate that the pH-5 enzyme fraction isolated by the method used in this experiment is not a simple protein.

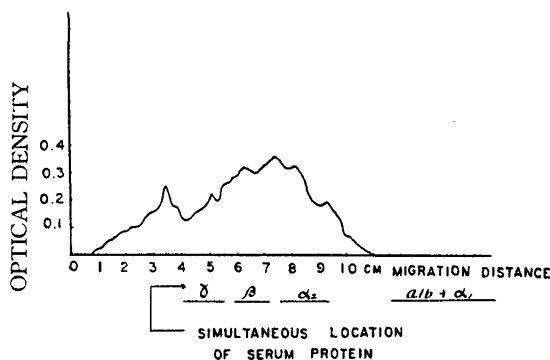
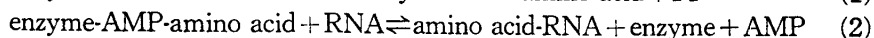
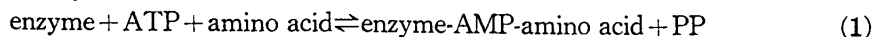


Fig. 1. Paper electrophoretic pattern of pH-5 enzyme.

DISCUSSION

Processes of protein synthesis in mammalian system are for convenience classified into three steps. The first step is carboxyl amino acid activation by pH-5 enzyme with simultaneous PP^{32} exchange reaction. The second is transferring the activated amino acid to pH-5 RNA. The third is incorporation of amino acid combined with pH-5 RNA to the microsomal protein. The formation shown in equation (1) and (2) represents the reaction of the first and second steps respectively.



The author examined the effect of PCMB on the first step and overall reaction of the first and second steps. On the first step, the inhibition of PCMB on the PP^{32} -ATP exchange occurring with leucine and restoration by cysteine may indicate that the leucine activating enzyme requires SH group for their activity.

The results are in good agreement with those of SCHWEET¹⁰ and OGATA *et al*¹¹. On the overall reaction of the first and second steps, it is recognized that labeling of S-RNA with C¹⁴-glycine by liver pH-5 enzyme may also be inhibited by PCMB. Namely, the inhibitory rate estimated at the end of the second step with PCMB is a little greater but almost similar compared with that of the first step on the assumption that the carboxyl activation of leucine is inhibited by PCMB in a similar degree as that of glycine. This fact is probably due to the decreased amount of activated amino acid caused by the first step inhibition. As to the third step, it will be also effected by the first and second reactions. That is, the limiting factor of inhibition of PCMB on protein biosynthesis will be a less amount of activated amino acids effected by the inhibition of SH active pH-5 enzyme by mercuric compounds. The effect of mercuric compounds to amino acid transferring systems has not been elucidated in this experiment.

Organic mercury compounds^{15,16}, i. e. phenyl mercury acetate and ethyl mercury phosphate, are widely used as agricultural agents, and induce severe poisoning on workers in the factory manufacturing these agents. These mercury compounds probably inhibit the processes catalysed by SH active respiratory enzymes (i. e. succinic dehydrogenase) and also amino-acid activation by SH active pH-5 enzyme, resulting in acute and chronic poisoning in mammalian systems, and the mechanism of effect of insect by PCMB will probably be the same as that of mammalian systems.

CONCLUSION

The labeling of C¹⁴-glycine to pH-5 enzyme, PP³²-ATP exchange by pH-5 enzyme, and the inhibitory action of p-chloromercuribenzoate were examined.

1) The labeling of C¹⁴-glycine to pH-5 enzyme is inhibited by 10⁻⁴ mole of PCMB, and this inhibitory effect is reduced by addition of cysteine having 20 mole equivalent of PCMB.

2) The PP³²-ATP exchange reaction is also reduced by addition of PCMB and inhibitory effect of PCMB is reduced by the addition of cysteine having 20 mole equivalent of PCMB.

3) These results show that the inhibition of the organic mercury compounds on the protein synthesis is responsible for attack the SH enzyme which catalyzes some amino-acid activation, and consequently influences activated amino-acid transfer reaction.

4) Paper electrophoretic pattern of pH-5 enzyme shows numerous peaks, each having the mobility between α_2 -globulin and γ -globulin.

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