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STUDIES ON THE BIURET-HYDROLYZING ENZYME FROM *MYCOBACTERIUM RANAE**

HIROMU NISHIHARA, KO SHOJI and MITSUO HORI

Department of Tuberculosis, Research Institute for Microbial Diseases, Osaka University, Osaka

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SUMMARY A newly found enzyme, tentatively named the biuret-hydrolyzing enzyme or biuretase, was isolated from *Mycobacterium ranae* and purified 28-fold. The properties of this enzyme were investigated in detail and the following results were obtained.

1) The optimum pH of the enzyme is about 9.5.
2) The most purified enzyme preparation is strictly specific for biuret, among the compounds tested.
3) Heavy metal ions, such as Ag^{+}, Hg^{2+}, Pb^{2+} and Mn^{2+}, inhibit the enzymatic activity more than 85 per cent.
4) A low concentration of *p*-chloromercuribenzoate completely inactivates the enzyme, and its activity is restored by addition of glutathione or cysteine. These results suggest that a sulfhydryl group is essential for the enzyme activity.
5) Although magnesium ion has not been confirmed to be an activator, it shows a slight activating effect on the enzymatic activity and restores the activity after inhibition by ethylenediaminetetraacetate.
6) The Michaelis constant of this enzyme was calculated to be 6.1×10^{-4} M.
7) Results indicate that biuret is decomposed by the enzyme into ammonia, carbon dioxide and urea.

INTRODUCTION

During investigation of the decomposition of amide compounds by mycobacteria, we found that suspensions of some mycobacterial strains (*Mycobacterium ranae*, *Mycobacterium avium*, *strain A*-62 and an *unclassified mycobacteria*, *strain Yamamoto-S*) were able to decompose biuret and liberate ammonia. Previously, the only report on the enzymatic decomposition of biuret has been on its slow decomposition by crystalline urease (SHAW and KISTIAKOWSKY, 1950). Our studies (SHOJI and NISHIHARA, 1962) on the biuret-hydrolyzing reaction using *M. avium*, *strain A*-62, which had no urease activity, indicated that there is a specific enzyme (the biruret-hydrolyzing enzyme or biuretase)

* An outline of this work was reported at the 39th Meeting of the Japanese Society for Tuberculosis on April 6, 1964, in Tokyo.

which is distinct from urease. About five months after our report, WHELDON and MACDONALD (1962) reported that *Pseudomonas aeruginosa* could utilize biuret as a sole nitrogen source, and that the biuret-hydrolyzing enzyme was inducible. However, they scarcely referred to the properties of this enzyme.

The present study is on the purification and properties of the enzyme of *M. ranae* with the hope of obtaining new enzymological and bacteriological findings.

MATERIALS AND METHODS

1. *Organism*

M. ranae was used throughout this work. It was both biuret- and urea-hydrolyzing activity. *M. avium, strain A*-62, used in the previous work, was not employed because it was found to lose its biuret-hydrolyzing activity from some unknown reason.

2. *Culture medium*

The medium used in this work contained the following constituents in distilled water: Meat extract (Wakô) 1 per cent, polypeptone (Wakô) 1 per cent, NaCl 0.3 per cent and glycerol 4 per cent. The pH was adjusted to 6.6 with NaOH. The medium was sterilized in an autoclave (120°C, 20 minutes).

3. *Chemicals*

All chemicals employed in the present investigation were of analytical grade. Biuret ($NH_2CONHCONH_2$, molecular weight 103.09) and urease (from Jack beans) were obtained from Wakô Pure Chemical Industries (Osaka), and the biuret was used without recrystallization.

4. *Measurement of biuret-hydrolyzing enzyme activity*

The enzyme activity was determined by estimation of NH_3 formation by Conway's micro-diffusion method (1933) and Russel's indophenol method (1944) modified by IMAI (1958), using solutions of recrystallized ammonium sulfate as standards. A Hitachi EPU-2 Beckmann type spectrophotometer was used in the colorimetric assay procedures. The standard reaction mixture was composed of biuret (0.005 M), pH 9.5 glycine-NaOH buffer (0.05 M) and enzyme in a total volume of 2.0 ml. To prevent release of NH_3 from the reaction mixture into the air during the incubation period, a small Thunberg tube was employed, containing the reaction mixture in the main chamber and 0.5 ml of 2 N H_2SO_4 solution in the side arm. Incubation was carried out at 37°C for 30 minutes. Then the H_2SO_4 solution was poured from the side arm into the main chamber and the mixture was shaken to stop the reaction. Then samples of 0.5 ml were withdrawn for estimation of NH_3. All estimations were made in duplicate or triplicate.

5. *Definitions of enzyme unit and specific activity*

Under the experimental conditions described above, the amount of enzyme which liberates one micromole of NH_3 in 30 minutes is defined as one unit of enzyme.

The specific activity is expressed as units per milligram of protein.

6. *Assay of protein content*

Determination of protein concentration was made by Lowry's method (1953), using crystalline bovine serum albumin as a standard.

7. *Preparation of the bacterial suspension*

Cells grown on glycerol broth were collected on a Buechner funnel and washed well with cold distilled water using a suction pump. The washed cells were blotted with filter papers to remove the moisture and weighed. Using glass beads, a homogeneous suspension in distilled water was prepared from them. This suspension was centrifuged at 3,000 rpm for 10 minutes and the resulting supernatant was discarded. This procedure was repeated at least three times until the supernatant did not give a color with Nessler's reagent. Finally, the cells were resuspended in distilled water at a concentration of approximately 20 mg per ml (wet weight). The dry weight of the suspension was estimated by drying a measured volume to constant weight at 120°C.

8. *Preparation of calcium phosphate gel*

Calcium phosphate gel was prepared by the method of KEILIN and HARTREE (1951). The dry weight of the gel suspension was about 13 mg per ml.

9. *Zone-electrophoresis*

Zone-electrophoresis using potato starch as a supporting medium was applied as a last step in the purification procedure following the method of YONEDA and FUKUI (1961), originally described by KUNKEL and SLATER (1952). Zone-electrophoresis was carried out in borate phosphate buffer, pH 8.3, ionic strength 0.1 under constant conditions with a potential gradient of 2 mA/cm² for 15 hours.

After electrophoresis, the starch block was cut into segments of 1 centimeter width and each segment was eluted twice with a 5 ml aliquot of 0.1 M buffered

saline (pH 7.0). Each eluate was filtered through fluted filter paper, and its activity and protein content were assayed.

RESULTS

1. *Variation in the biuret- and urea-hydrolyzing activities during cultivation of M. ranae*

Volumes of 80 ml of glycerol broth medium in 300 ml Erlenmeyer flasks were autoclaved. Then one loopful of the pellicle of a 4 day old culture in glycerol broth was inoculated into each flask and the flasks were incubated at 37°C. At intervals, the cells of two flasks were collected and a bacterial suspension was prepared from each as described in the MATERIALS AND METHODS section.

The biuret- and urea-hydrolyzing activities of the bacterial cells were expressed as micromoles of NH_3 formed per 2 hours per milligram dry weight of bacilli. The following assay systems were used: For measurement of biuret-hydrolyzing activity, biuret (0.005 M), pH 9.5 glycine-NaOH buffer (0.05 M) and 0.5 ml of bacterial suspension in a total volume of 2.0 ml. For urea-hydrolyzing activity, urea (0.005 M), pH 7.5 phosphate buffer (0.05 M) and 0.5 ml of bacterial suspension in a total volume of 2.0 ml of reaction mixture. The yield of bacilli was expressed as grams dry weight.

As shown in Fig. 1, the growth of *M. ranae* on glycerol broth medium reaches a maximum in 4 days. Urea-hydrolyzing activity is greatest in the early stage of cultivation, and then decreases with time. On the contrary, the biuret-hydrolyzing activity increases with the cultivation time and reaches a maximum on the 8th day. These findings strongly suggest that the biuret-hydrolyzing reaction is catalyzed by a specific enzyme which is distinct from urease. These results also indicate that an 8 day old culture is the most suitable for extraction of biuretase.

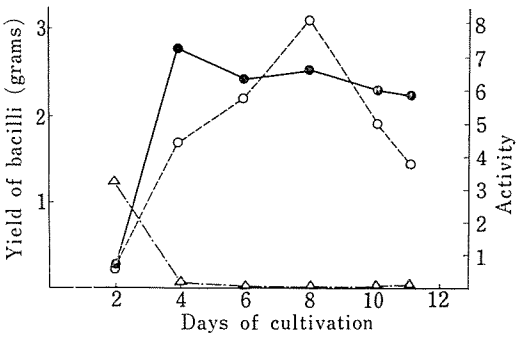


FIGURE 1 Variation in the Biuret- and Urea-Hydrolyzing Activities during Cultivation of *M. ranae*.
●——● yield of bacilli
○- - - - - -○ biuret-hydrolyzing activity
△——△ urea-hydrolyzing activity
Medium : 4 per cent glycerol broth medium.
Details are described in the text.

2. *Distribution of the biuret-hydrolyzing enzyme in subcellular fractions*

After 8 days culture on glycerol broth medium cells of *M. ranae* were ground in a mortar with an equal volume of quartz sand and extracted with 5 volumes of 0.05 M phosphate buffer (pH 7.5), and then the extract was fractionated by centrifugation. Table 1 shows that biuretase activity is present in the supernatant obtained by centrifugation at 105,000×*g* for 60 minutes, that is, in the soluble fraction.

TABLE 1 *Distribution of biuret-hydrolyzing enzyme in subcellular fractions*

Subcellular fraction	Total protein mg	Total units	Specific activity units/mg	Recovery %
1. Supernatant after centrifugation at 900×g, 20 min.	29.66	113.60	3.8	100
2. Supernatant after centrifugation at 14,500×g, 40 min.	25.60	100.86	3.9	90.0
3. Precipitate after centrifugation at 14,500×g, 40 min.	0.90	0	0	0
4. Supernatant after centrifugation at 105,000×g, 60 min.	22.42	91.92	4.1	88.8
5. Precipitate after centrifugation at 105,000×g, 60 min.	0.78	0	0	0

3. *Purification of the biuret-hydrolyzing enzyme*

All procedures were carried out in the cold.

Step 1. *Preparation of the crude extract*

An 8 day old culture of *M. ranae* grown on glycerol broth medium (1,220 g, wet weight) was collected and washed with cold distilled water. The washed cells were ground in a mortar for one hour with an equal volume of quartz sand and extracted with 2 volumes of 0.05 M phosphate buffer (pH 7.5). The extract was centrifuged at $8,100 \times g$ for 20 minutes. The precipitate was reextracted with 0.5 volume of the same buffer and the supernatant obtained by centrifugation was combined with the previous supernatant. The combined supernatant was then filtered through fluted filter paper and centrifuged at 14,500 $\times g$ for 30 minutes. The resulting supernatant was again filtered through filter paper and the filtrate was dialyzed against 0.05 M phosphate buffer (pH 7.5). A crude extract of 2,548 ml was obtained (protein concentration 6.02 mg/ml).

Step 2. *Treatment with protamine sulfate*

An approximately 1 per cent solution of protamine sulfate neutralized with NaOH was prepared. The amount of protamine solution required to remove nucleic acid completely was determined on a 5 ml sample of the crude extract. One-fourth of this quantity (38.1 ml of protamine solution) was added to the crude extract of 2,543 ml, because the biuretase protein had a tendency to precipitate by conjugation with protamine on addition of a large quantity of protamine. The precipitate was removed by filtration and 2561 ml of filtrate was obtained (protein concentration 4.50 mg/ml).

Step 3. *First fractionation with ammonium sulfate*

To the filtrate from the above step, saturated ammonium sulfate solution adjusted to pH 6.5–7.0 with NH_3 was added to give 30 per cent saturation. After standing the mixture overnight in the cold, the precipitate was removed by centrifugation. The second precipitate, obtained by increasing the ammonium sulfate concentration to 50 per cent saturation and standing the mixture overnight, was collected by centrifugation, dissolved in 0.05 M phosphate buffer (pH 7.5), and dialyzed against the same buffer for 2 days. A slightly turbid brown solution of 71.3 ml was obtained (protein concentration 27.40 mg/ml).

Step 4. *Adsorption of impurities by calcium phosphate gel*

The enzyme preparation obtained in step 3 was mixed with 0.3 volume of calcium phosphate gel, prepared by Keilin's method. After stirring the mixture for 60 minutes, the gel was removed by centrifugation. A brownish solution of 102 ml (protein concentration 13.20 mg/ml) was obtained.

Step 5. *Second fractionation with ammonium sulfate*

The supernatant fluid from the preceding step was refractionated with saturated ammonium sulfate solution by the same method as in step 3. A clear dark brown solution of 14.8 ml (29.80 mg/ml) was obtained.

Step 6. *Zone-electrophoresis*

As a last step in the purification, zone-electrophoresis using potato starch as supporting medium was employed as described in the

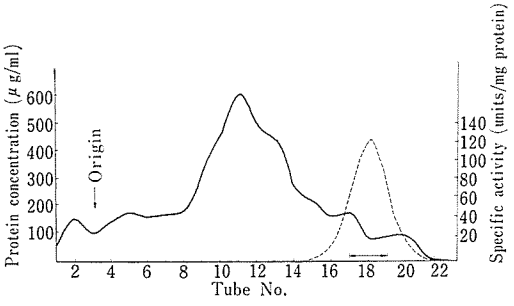


FIGURE 2 Zone-Electrophoretic Pattern of the Enzyme Preparation Obtained in Step 5.

——— protein concentration

- - - - - - - - - specific activity

Condition: Borate phosphate buffer (pH 8.3, ionic strength 0.1), electric current 2 mA/cm², 15 hours, protein concentration 1.38 per cent (5 ml)

The eluates of tube Nos. 17–19 were collected and concentrated with $(NH_4)_2SO_4$.

MATERIALS AND METHODS section. The preparation in step 5 was dialyzed overnight against borate phosphate buffer (pH 8.3, ionic strength 0.1), diluted with the same buffer to a concentration of 1–2 per cent protein and 5 ml of the diluted dialyzate was charged on a starch block prepared in a plastic cell ($7 \times 40 \times 1.5$ cm). It had been confirmed that there was no appreciable loss of activity due to adsorption on the potato starch.

In all cases an electrophoretic pattern similar to that shown in Fig. 2 was obtained. Fractions of high activity had moved far to the anodic side. The most purified fraction had about 78-fold the specific activity of the crude extract. The eluates in tube Nos. 17–19, which were highly purified, were collected in a cellophane bag and dialyzed against 70 per cent saturated ammonium sulfate solution for 2 days.

The resulting precipitate was collected by centrifugation at $14{,}500 \times g$ for 30 minutes, dissolved in 0.05 M phosphate buffer (pH 6.0), and dialyzed for 2 days against the same buffer. A clear white preparation of 10.4 ml (protein concentration 2.7 mg/ml) was obtained. This preparation had been purified 28-fold, as shown in Table 2, but its yield of 5.2 per cent was not satisfactory.

The preparation in step 6 lost about 50 per cent of its original activity within a few days at −20°C. Addition of glutathione or cysteine to the preparation had little effect on its stability. The ultraviolet absorption spectra of this preparation had a characteristic peak at 280 mμ identical with that of a pure protein solution. The nucleic acid content in the most purified preparation calculated from the 280/260 mμ ratio (WARBURG and CHRISTIAN, 1941) was 0.9 per cent.

Though high activities of amidases acting on formamide, acetamide and acrylamide were found in the crude extract, the purified enzyme preparation in step 6 did not have these activities, indicating its purity.

The preparations obtained in steps 5 and 6 were employed in the following experiments.

TABLE 2 *Summary of purification procedures*

Step	Total protein mg	Total units	Specific activity units/mg	Purification	Yield %
1. Crude extract	15,300.3	26,014.9	1.7	1	100
2. Protamine treatment	11,524.5	24,667.5	2.1	1.2	94.8
3. First $(NH_4)_2SO_4$ fractionation 30-50% saturation	1,954.0	17,586.1	9.0	5.3	67.6
4. Calcium phosphate gel treatment	1,345.8	13,996.0	10.4	6.1	53.8
5. Second $(NH_4)_2SO_4$ fractionation 30-50% saturation	441.2	8,250.2	18.7	11.0	35.6
6. Zone-electrophoresis $(NH_4)_2SO_4$ precipitation	28.4	1,352.8	47.6	28.0	5.2

4. *Properties of the biuret-hydrolyzing enzyme*

1) *Optimal pH*

The effect of pH on the enzyme activity was

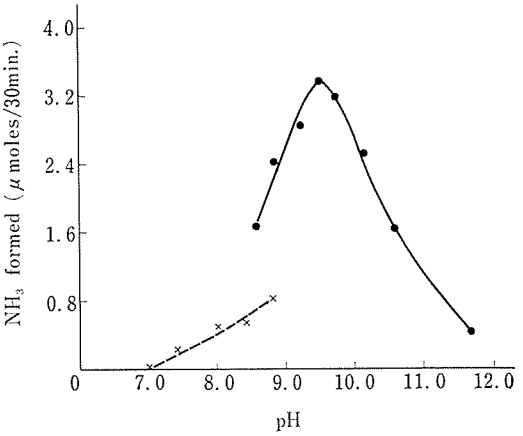


FIGURE 3 Effect of pH on Biuret-Hydrolyzing Enzyme Activity

●——● in 0.05 M glycine-NaOH buffer

×- - - - - - - × in 0.05 M Tris-HCl buffer

Reaction mixture: Biuret (0.005 M), buffer (0.05 M), enzyme (at step 6, 72 μg protein); total volume 2.0 ml

investigated with the most purified enzyme preparation. As seen in Fig. 3, the pH-activity curve is fairly sharp and the pH optimum in 0.05 M glycine-NaOH buffer is nearly 9.5. Biuretase shows stronger activity in glycinate buffer than in tris (hydroxymethyl) aminomethane (Tris)-HCl buffer, as seen from its activity between pH 8.6 and 8.8.

2) *Heat stability of the biuret-hydrolyzing enzyme*

The heat stability of this enzyme was examined with preparations at steps 5 and 6. As shown in Fig. 4, both preparations are fairly stable on being heated at 50°C for 5 minutes. However on being heated at 60°C for 10 minutes, the preparation at step 6 loses about

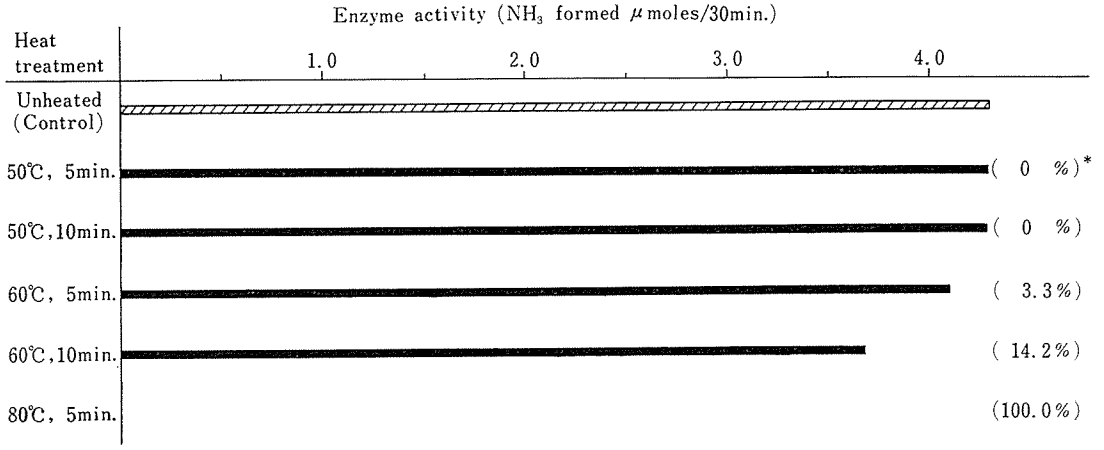


FIGURE 4 a) Heat Stability of Biuret-Hydrolyzing Enzyme. a) Enzyme Preparation at Step 5.
Heat treatment was carried out in 0.05 M phosphate buffer (pH 7.5) at a protein concentration of 0.48 mg/ml. After the treatment, the preparations were cooled in ice water, and enzyme activity was assayed. The reaction mixture (2.0 ml) was composed of biuret (0.005 M), pH 9.5 glycinate buffer (0.05 M) and enzyme (0.24 mg/ml protein).
* Numbers in parenthesis indicate % loss of activity.

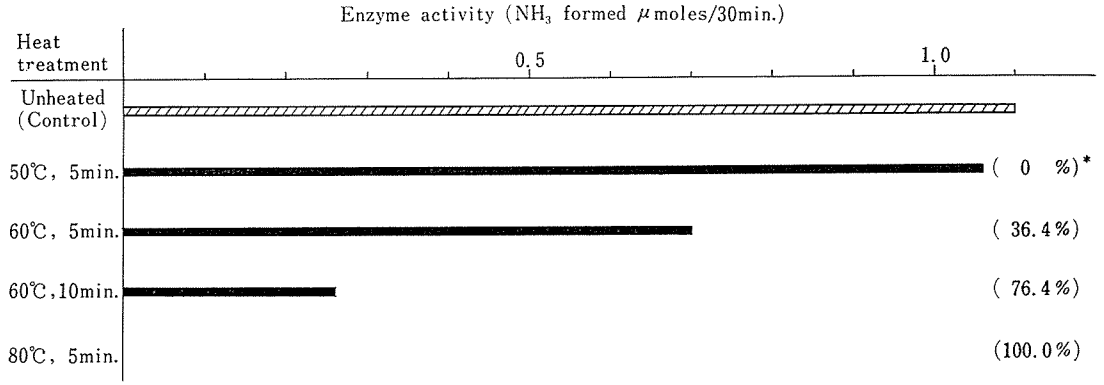


FIGURE 4 b) Heat Stability of Biuret-Hydrolyzing Enzyme. b) Enzyme Preparation at Step 6.
Enzyme preparations were heated in 0.05 M phosphate buffer (pH 6.0) at a protein concentration of 61 μg/ml. Then the preparations were cooled in an ice bath, and enzyme activity was assayed. The reaction mixture (2.0 ml) consisted of biuret (0.005 M), pH 9.5 glycinate buffer (0.05 M) and enzyme (31 μg/ml protein).
* Numbers in parenthesis indicate % loss of activity.

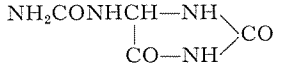
76 per cent of its activity, whereas the preparation at step 5 is comparatively stable (about 14 per cent loss of activity). After 5 minutes at 80°C, both preparations lose their activity completely.

Both preparations were most stable at about pH 6.0, but at below pH 4.0 they were inactivated rapidly.

3) *Substrate specificity*

Among the compounds tested, the most purified enzyme preparation had absolute substrate specificity, as shown in Table 3.

TABLE 3 *Substrate specificity of biuret-hydrolyzing enzyme*

Substrate	Chemical structure	Enzyme activity*
Biuret	$NH_2CONHCONH_2$	4.06
Urea	NH_2CONH_2	0
Malonamide	$NH_2COCH_2CONH_2$	0
N-Acetylurea	$CH_3CONHCONH_2$	0
Allophanic acid ethylester	$NH_2CONHCOOC_2H_5$	0
Carbamic acid (Na salt)	NH_2COONa	0
Formamide	$HCONH_2$	0
Acetamide	CH_3CONH_2	0
Acrylamide	$CH_2CHCONH_2$	0
Oxamide	$NH_2COCONH_2$	0
Succinamide	$NH_2COCH_2CH_2CONH_2$	0
1,3-Dimethylurea	$CH_3NHCONHCH_3$	0
Cyanoacetamide	$CNCH_2CONH_2$	0
1-Acetyl-2-thiourea	$CH_3CONHCSNH_2$	0
Thiosemicarbazide	$NH_2NHCSNH_2$	0
Isobutyramide	$(CH_3)_2CHCONH_2$	0
Allantoin		0
Phenylurea	$C_6H_5NHCONH_2$	0

* Enzyme activity was expressed as μ moles of NH_3 formed per 30 min.
Reaction mixture: Substrate (0.01 M), pH 9.5 glycinate buffer (0.05 M) enzyme (at step 6, 90μg protein); total volume 2.0 ml

Malonamide and N-acetylurea, closely analogous in structure to biuret, are not utilized as substrates. It was also confirmed that this enzyme is not identical with urease, because urea is not hydrolyzed by the purified preparation.

4) *Inhibition of the biuret-hydrolyzing enzyme by various agents*

The following experiments were performed with the preparation at step 5, and the results are shown in Table 4.

Heavy metal ions such as Ag^+, Hg^{2+}, Pb^{2+} and Mn^{2+} cause more than 85 per cent inhibition of the enzyme activity. At a concentration of 2.5×10^{-4} M, magnesium ion causes slight activation, but has no effect at a concentration of 5.0×10^{-3} M. However, the degree of activation by magnesium ion varied with the experimental conditions. Hydroquinone which strongly inhibits urease also inhibits biuretase strongly. The enzyme is inhibited completely by *p*-chloromercuribenzoate (PCMB) at a concentration of more than 2.0×10^{-4} M. Ethylenediaminetetraacetate (EDTA) causes 20–30 per cent inhibition, but the degree of inhibition varied in different enzyme preparations. Urea and malonamide tested as possible competitive inhibitors, caused inhibitions of about 18 and 11 per cent, respectively. However, to decide whether these compounds are actually competitive in-

TABLE 4 *Inhibition of biuret-hydrolyzing enzyme by various agents*

Inhibitor	Inhibitor concentration Mole	NH_3 formed μ moles/30min.	Inhibition %	(Activation) %
None (control)	0	3.95		
$CuCl_2$	2.5×10^{-3}	1.51	61.8	
$AgNO_3$	2.5×10^{-4}	0.03	99.3	
$HgCl_2$	,,	0	100	
$Pb(CH_3COO)_2$	,,	0.19	95.3	
$MnSO_4$	,,	0.67	85.3	
$ZnCl_2$	,,	1.74	55.9	
$NiCl_2$	,,	3.06	22.5	
$CoCl_2$	,,	3.22	18.5	
$CaCl_2$	,,	3.91	1.0	
$MgCl_2$	5.0×10^{-3}	3.95	0	
	2.5×10^{-4}	4.87		(23.3)
Hydroquinone	1.0×10^{-3}	0.23	94.3	
Sodium salicylate	,,	3.21	18.7	
Guanidine hydrochloride	,,	3.57	9.6	
p-Chloromercuribenzoate	,,	0	100	
	5.0×10^{-4}	0	100	
	2.0×10^{-4}	0	100	
	1.0×10^{-4}	2.83	26.9	
	1.0×10^{-5}	3.12	20.9	
Ethylenediaminetetraacetate	1.0×10^{-3}	3.00	24.1	
	1.0×10^{-4}	2.82	28.5	
Urea	1.0×10^{-2}	3.22	18.4	
Malonamide	,,	3.50	11.4	

Reaction mixture: Biuret (0.005 M), pH 9.5 glycine-NaOH buffer (0.05 M), enzyme (at step 5, 0.23mg protein), inhibitor; total volume 2.0 ml.

TABLE 5 *Reactivation of biuret-hydrolyzing enzyme inactivated by PCMB and restoration of inhibition by EDTA*

Inhibitor	NH_3 formed μ moles/30 min.	Inhibition %	Reactivator		NH_3 formed μ moles/30 min.	Reactivation %
Control	3.95					
PCMB 2.0×10^{-4} M	0	100	Glutathione	1.5×10^{-2} M	4.40	111.4
1.0×10^{-3} M	0	100		2.0×10^{-2} M	4.84	122.5
2.0×10^{-4} M	0	100	Gysteine	1.5×10^{-2} M	4.36	110.5
1.0×10^{-3} M	0	100		2.0×10^{-2} M	5.70	144.3
EDTA 1.0×10^{-3} M	3.00	24.1	$MgSO_4$	1.0×10^{-2} M	3.95	100.0

Reaction mixture: Biuret (0.005 M), pH 9.5 glycine-NaOH buffer (0.05 M), enzyme (at step 5, 0.23 mg protein), inhibitor, (with or without activator); total volume 2.0 ml.
Reactivation test: Enzyme solutions (0.16 mg/ml protein) were preincubated with the inhibitors at 37°C for 5 minutes, and then the activators were added and the enzyme activity was measured.

hibitors, studies on the kinetics of a more purified enzyme preparation will be necessary.

5) *Reactivation of the biuret-hydrolyzing enzyme after inactivation by PCMB and restoration of the inhibition by EDTA*

The inactivation caused by PCMB is completely overcome by addition of glutathione or cysteine, as seen in Table 5. It is suggested from these data that the sulfhydryl group may be essential for the activity of the biuret-hydrolyzing enzyme, as it is in urease. The inhibition caused by EDTA is also reversed by addition of magnesium ion of 10-fold of the concentration of the EDTA. However, it is uncertain whether the enzyme requires Mg ion for its activity, because both the inhibition caused by EDTA and the activation caused by Mg ion were slight. More detailed investigations are in progress on this problem.

6) *Substrate concentration and Michaelis constant*

The influence of substrate concentration on enzyme activity was investigated using the purest preparation. The relationship between the initial velocity and the substrate concentration is shown in Fig. 5. The Michaelis-Menten constant calculated from these data by a Lineweaver-Burk plot is 6.1×10^{-4} M.

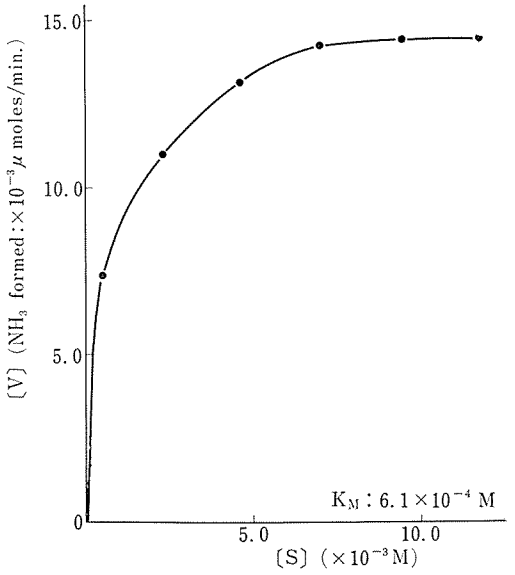


FIGURE 5 Effect of Substrate Concentration on Biuret-Hydrolyzing Enzyme Activity.
Enzyme at step 6 was used. The Michaelis constant was calculated from a Lineweaver-Burk plot.

7) *Mode of action of the biuret-hydrolyzing enzyme*

Biuret could be decomposed by biuretase by one of the following three reactions:

I) $NH_2CONHCONH_2 \xrightarrow{+2H_2O} 3\,NH_3+2\,CO_2$

II) $NH_2CONHCONH_2 \xrightarrow{+H_2O} NH_3+NH_2CONHCOOH$ (allophanic acid)

III) $NH_2CONHCONH_2 \xrightarrow{+H_2O} NH_3+CO_2+NH_2CONH_2$ (urea)

Stoichiometric analysis should be made to determine which of these reactions in fact occurs, but it was impossible to find an easy and specific assay method for biuret, so that indirect analysis had to be used. The following points are significant in deciding which of the three reactions actually occurs:

(1) Only in reaction I is the molar evolution of NH_3 greater than the molar concentration of biuret initially present in the reaction mixture.

(2) If additional ammonia is formed on addition of urease to the reaction mixture at the end of the reaction, reaction III has probably occurred.

(3) If reaction III occurs, the molar ratio of NH_3 to CO_2 should be one.

(4) In reaction II no CO_2 evolution should be observed unless the allophanic acid is subsequently decomposed.

Taking these assumptions into consideration, the following experiment was carried out: Using an initial concentration of biuret of 2 μ moles/ml in a total volume of 4 ml, the amount of NH_3 liberated from the biuret was followed during an incubation period of 150

minutes by assay of 0.5 ml aliquots at various intervals. At the same time, the amount of NH_3 released from the reaction mixture into the air, although very small, was checked by an experiment in a Thunberg tube. After the 150 minute incubation period, the reaction mixture was heated at 100°C for 10 minutes to stop the reaction, and then an equal volume of urease solution was added to the reaction mixture at a final concentration of 2 mg/ml. Then the mixture was reincubated to allow for further ammonia formation. As shown in Fig. 6, the amount of ammonia produced during the first incubation period was not more than the initial biuret concentration of 2 μmoles per ml, and much ammonia formation was observed on addition of urease. These results suggest that biuret is decomposed through reaction III.

To confirm the above conclusion, a further

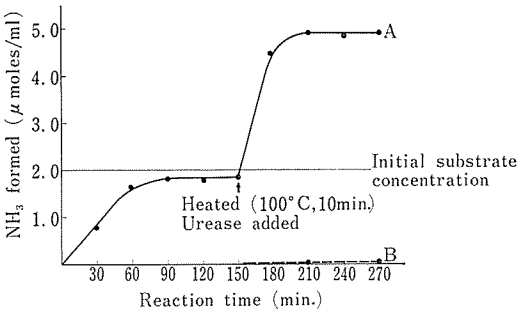


FIGURE 6 Mode of Action of the Biuret-Hydrolyzing Enzyme.
Details are described in the text.
Reaction mixture of A: Biuret (2 μ moles/ml), pH 9.5 glycinate buffer (0.05 M), enzyme (at step 6, 680 μg protein); total volume 4.0 ml
Reaction mixture of B: Biuret (2 μ moles/ml), pH 9.5 glycinate buffer (0.05 M), urease (2 mg/ml); total volume 2.0 ml

TABLE 6 *Quantitative analysis of ammonia and carbon dioxide liberation from biuret by biuret-hydrolyzing enzyme*

Experiment	Reaction time minutes	NH_3 formed* μ moles	CO_2 evolution** μ moles	NH_3/CO_2
1.	10	1.35	1.13	1.19
	20	1.77	1.47	1.20
	60	5.28	6.21	0.85
2.	40	2.73	2.63	1.04
	50	3.78	3.25	1.16
	80	6.66	4.83	1.38
3.	20	2.85	4.10	0.70
	30	3.90	5.28	0.74
	,,	4.08	5.01	0.81
	40	6.51	6.22	1.05
4.	20	1.47	1.86	0.79
	30	2.46	2.44	1.01
	45	3.96	3.39	1.17
	60	5.28	5.10	1.04
	70	5.55	6.43	0.86

* Ammonia was measured by Conway's micro-diffusion method and Russel's indophenol method modified by IMAI.

** Carbon dioxide was estimated by the Warburg manometric method. The reaction mixture in a Warburg flask was as follows:

Main chamber; pH 7.4 Tris-HCl buffer (0.01 M), 1.7 ml enzyme solution (at step 5), 0.5 ml
Side arm I. ; biuret solution (27 μ moles/ml), 0.5 ml
Side arm II. ; 4N H_2SO_4, 0.3 ml
total volume 3.0 ml, incubation at 37°C

experiment was carried out: The amounts of NH_3 and CO_2 produced were estimated at the same intervals during the incubation period.

Carbon dioxide was measured manometrically in a Warburg manometer (Umbreit *et al.*, 1951). However, alkaline buffers, such as pH 9.5 glycine-NaOH buffer, contain large quantities of CO_2, so there is much CO_2 evolution from the blank containing no enzyme. Therefore, 0.01 M Tris-HCl buffer (pH 7.4), which contained less than one-half of the CO_2 of glycinate buffer, was used. A high concentration of the partially purified enzyme preparation at step 5 had to be used, because the most purified preparation had low activity in Tris buffer, pH 7.4. Table 6 shows that the molar ratio of ammonia to carbon dioxide found was nearly one. On the basis of these results, it seems likely that one mole of biuret is hydrolyzed by biuretase into one mole each of ammonia, carbon dioxide and urea. However the possibility that allophanic acid might be formed as an intermediate product has not been excluded.

DISCUSSION

The previous report was on the biuret-decomposing reaction shown by some strains of mycobacteria. *M. avium, strain A*-62 was used and evidence for the existence of a new enzyme, tentatively named the biuret-hydrolyzing enzyme or biuretase, was presented. In the present work, this enzyme from *M. ranae* was purified and its properties were investigated with purified enzyme preparations. In addition, the absolute specificity of this enzyme to biuret has been established using the purified enzyme preparation. The purification procedures employed in this work, however, were unsatisfactory because the yield was poor. To find more suitable purification procedures, the stabilizing effects of various agents on biuretase were examined. The addition of glutathione or cysteine to the enzyme preparation had little effect on its stability. Recently it was shown that addition of EDTA, which exhibited a low degree of inhibition, to the enzyme preparation prevented the denaturation of biuretase, probably by chelating with heavy metal ions, some of which inhibit biuretase strongly. To obtain a better yield during the purification procedures, various methods, including column chromatography, are being investigated. Preliminary tests of Sephadex G-100 and G-200 column chromatography indicated that the molecular weight of biuretase is in the range of 100,000–200,000. The isoelectric point of the protein molecule of biuretase seems to be quite low because the enzyme is precipitated by protamine, and also because the enzyme moved rapidly to the anode during zone-electrophoresis. But most of the physicochemical properties of the enzyme are still unknown.

With regard to the mode of action of the biuret-hydrolyzing enzyme, it is concluded from the present results that biuret is decomposed by the enzyme into NH_3, CO_2 and urea. However, Wheldon *et al.* reported that with a preparation of fragmented cells of *Pseudomonas aeruginosa* biuret was hydrolyzed with the stoichiometric release of 3 moles ammonia per mole of biuret. This suggests that the properties of these two biuretases from different bacteria are not the same. In support of this, the pH optimum of the biuretase from *Pseudomonas* is 7.5, whereas that of the biuretase from *M. ranae* was found to be 9.5. The preparations used by Wheldon *et al.* were crude and were probably contaminated with a considerable amount of urease, so that the possibility that the two reactions overlapped cannot be ruled out, as they themselves pointed out.

The urea-hydrolyzing activity of *M. ranae* grown on glycerol broth medium decreased with the length of the cultivation period, while the biuret-hydrolyzing activity increased and reached a maximal level after 8 days (Fig. 1). These results suggest that there is some relation between urease and biuretase.

The role of the biuret-hydrolyzing enzyme in the metabolism of mycobacteria is still obscure: It has never been reported that biuret, an artificial derivative of urea, was a metabolite

or constitutent of bacteria. However the biuretase activity of mycobacteria was constitutive and addition of biuret to the medium did not cause an elevation of its activity. On the other hand, so far as tested, this enzyme is strictly specific for biuret. In view of these facts, the question arises of why this enzyme is synthesized constitutively, not inducibly. Studies on this problem are in progress.

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