This compound was identical with the one derived from the grisenone (XIIb) by mixed melting point determination and comparison of IR spectra.

The author is indebted to Prof. Emeritus S. Sugasawa of the Tokyo University and to the Director Dr. N. Sugimoto for their kind and helpful advice.

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Summary

4-(*o*-Methoxymethoxybenzoyl)-1,3-cyclohexanedione, having a *β*-tricarbonyl system, was synthesized by the interaction of disodio-1,3-cyclohexanediene and methyl *o*-methoxymethoxybenzoate in liquid ammonia.

4-(*o*-Hydroxybenzoyl)-1,3-cyclohexanedione, having a hypothetical *β*-tetracarbonyl system, was also prepared. Grisan-2′,3,4′-trione yielded from the *β*-tetraketone by means of alkaline ferricyanide oxidation. The structures of its two isomeric methyl enol ethers were proved by deriving them to grisan-2′,3- and grisan-3,4′-dione.

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31. Makoto Hayashi, Reiko Uchida, Tsutomu Unemoto, and Komei Miyaki : Enzymic Oxidation of Ethanolamine by Beef Serum.*1

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It has been already informed that there is an oxidase capable of oxidizing spermine, spermidine, several aliphatic and aromatic amines, and normal long-chain diamines in the sera of sheep, beef and goat.[1,2] Tabor, *et al.*,[2] using partially purified enzyme, elucidated the decomposition of primary monoamines and long-chain diamines. Yamada and Yasunobu[3] obtained an oxidase in the beef serum in a crystalline form.

One of authors[4] has already reported on the oxidation of polyamines, and during the process of this study it was found that the serum of beef or goat oxidatively decomposes alkanolamine, especially ethanolamine, besides afore-mentioned amines. Therefore, with the object of investigating whether the oxidase already reported and the ethanolamine oxidizing enzyme are identical or not, separation by DEAE-cellulose column chromatography was attempted after fractionation with ammonium sulfate. However, it was found that these oxidases could not be separated by the present method. The present paper describes the oxidation of ethanolamine by partially purified enzyme in beef serum.

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*1 This was presented at the 81st Annual Meeting of Pharmaceutical Society of Japan (July, 1961 in Sapporo).

*2 Okubo, Narashino, Chiba-ken (林　誠, 内田礼子, 畝本　力, 宮木高明).

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Experimental

Materials——Ethanolamine, N-methylethanolamine, N,N-dimethylethanolamine, 3-hydroxypropylamine and butylamine were obtained from Tokyo Kasei Kogyo Co. and used as respective hydrochloride prepared after distillation. Choline chloride and glyoxal were purchased from Tokyo Kasei Kogyo Co. and the latter was used after distillation. 4-Hydroxybutylamine was synthesized from 4-aminobutyrate in this laboratory. DL-2-Hydroxypropylamine hydrochloride, DL-3-hydroxy-4-aminobutyric acid, DL-3-hydroxyglutamic acid and dihydroxymaleic acid were offered by Dr. N. Ikeda, Faculty of Pharmacy, Chiba University. Glycolaldehyde was synthesized from dihydroxymaleic acid by the method of Powers, *et al.*[5] The other reagents used were those of special grade.

Enzyme Assay——An oxidation reaction was carried out at 37° and oxygen consumption was measured by Warburg apparatus. The ammonia formed was estimated by the method of Unemoto, *et al.*[6] after separation by Conway microdiffusion procedure. The reaction mixture consisted of 0.05*M* phosphate buffer (pH 7.5), 0.2 ml. of enzyme solution (see below) and a substrate in a total volume of 1.0 ml. The final concentration of substrate used for ethanolamine oxidation was $5\times10^{-3}M$ ethanolamine, that for polyamine oxidation was $10^{-3}M$ spermine, and that for monoamine oxidation was $5\times10^{-3}M$ butylamine. In a manometric method, total volume was made up to 2.0 ml. without changing the final concentrations of all components.

Enzyme Purification

1) **Fractionation of Beef Serum by Ammonium Sulfate**——$(NH_4)_2SO_4$ was added to a beef serum with stirring and cooling, and precipitates obtained at each saturation degree were fractionally separated. Each precipitate was dissolved in 0.05*M* phosphate buffer (pH 7.3) in a 0.1 volume of the original serum, which was then dialyzed at 5° against the same buffer until the amount of NH_3 in the interior solution became lower than 0.01 μmole per ml. As shown in Fig. 1, about 60% of each oxidase activity was found in the fraction saturated in the degree of 0.4~0.5 with $(NH_4)_2SO_4$. This fraction was used as a preparation for further purification.

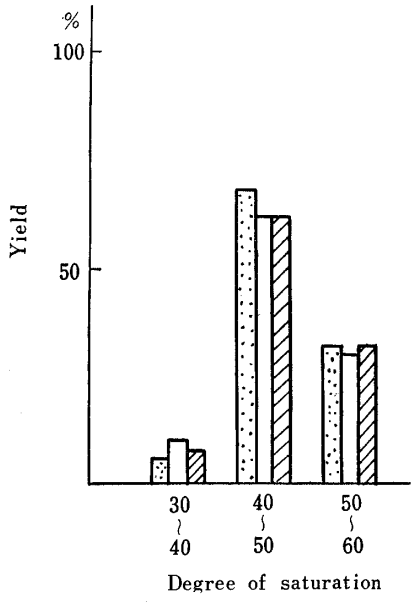

Fig. 1. Fractionation of Beef Serum by Ammonium Sulfate

The enzyme activity found in each fraction was expressed as per cent of total activity.

▒ ethanolamine as a substrate
□ spermine as a substrate
▨ butylamine as a substrate

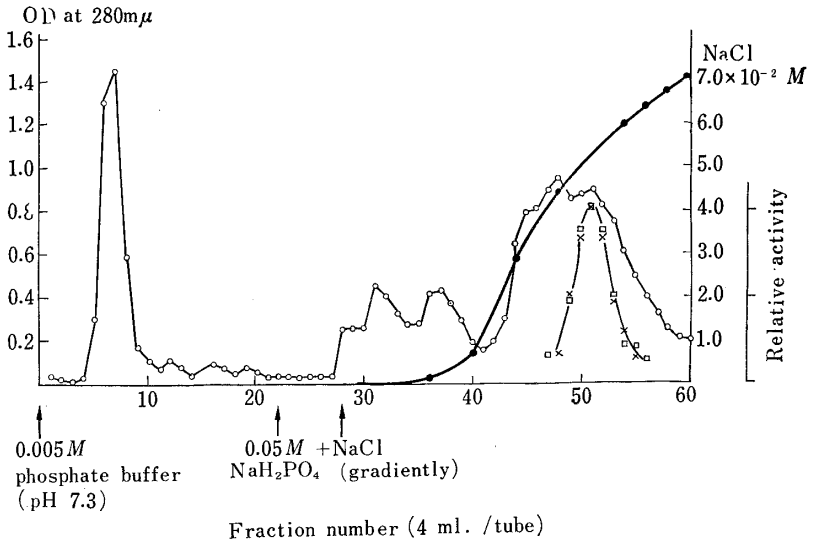

Fig. 2. DEAE-Cellulose Column Chromatography of Amine Oxidase in Beef Serum

For details of experimental conditions see text.
○—○ proteins (measured by the absorbancy at 280 mμ)
●—● concentration of NaCl in the eluate
□—□ oxidase activity (spermine as a substrate)
×—× oxidase activity (ethanolamine as a substrate)

5) H. H. Powers, G. Tabakoglu, H. Z. Sable : Biochem. Preparations, **4**, 56 (1955).
6) T. Unemoto, Y. Tsuda, M. Hayashi : Yakugaku Zasshi, **80**, 1089 (1960).

2) **Purification by DEAE-Cellulose Column Chromatography**——Above mentioned sample (in an amount corresponding to 150 ml. of original serum) was charged on a DEAE-cellulose column (1 × 40 cm.) previously bufferized with 0.005*M* phosphate buffer (pH 7.3), and the proteins adsorbed on the column were eluted successively with 0.005*M* phosphate buffer (pH 7.3), 0.05*M* NaH_2PO_4, and then 0.05*M* NaH_2PO_4 plus NaCl. NaCl was added in a gradient manner and its concentrations in the eluates were indicated in Fig. 2. Elution diagram of the proteins and of enzyme activity were illustrated in Fig. 2. Fractions having enzyme activity were combined and the concentration of protein was adjusted to 1 mg. per ml. This was used as an enzyme preparation throughout the experiments.

Results

Optimum pH and Apparent Km

As shown in Fig. 3, the optimum pH was 7.5. The reaction velocity increased with the increase in the concentration of ethanolamine and the apparent Km was about $4\times 10^{-3}M$ (Fig. 4).

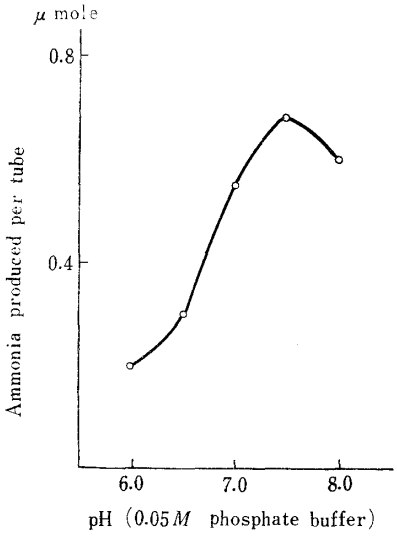

Fig. 3. pH-Activity Curve of Ethanolamine Oxidation

Reaction mixture (1.0 ml.) was incubated for 1 hr. For experimental conditions, see test.

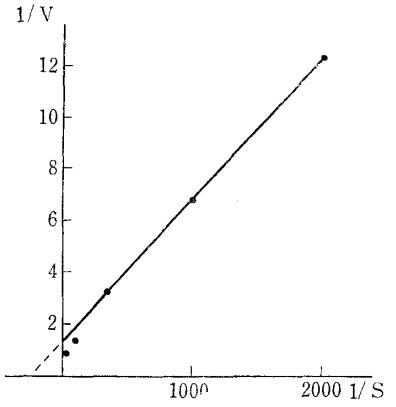

Fig. 4. Lineweaver and Burk Plot

Reaction mixture (1.0 ml.) was incubated for 30 min. For experimental conditions, see text. V was calculated from the amount of ammonia produced per tube. S denotes the final concentration of ethanolamine in the reaction mixture.

Oxygen Consumption and Ammonia Formation

As indicated in Fig. 5, oxygen consumption and ammonia formation increased in parallel and the molecular ratio of the amount of oxygen consumed and the amount of ammonia formed was 1:1. Since no catalase was certainly contained in this enzyme preparation, it was supposed that reaction might follow the general reaction mechanism of oxidase as given below.

$$RCH_2NH_2 \xrightarrow{O_2} RCHO + NH_3 + H_2O_2 \xrightarrow{\text{catalase}} \tfrac{1}{2}O_2 + H_2O$$

However, even on adding purified catalase either prior to the reaction or during the reaction, the ratio of O_2: NH_3 was always kept at about 1:1. As it has been well known that catalase behaves like peroxidase in the presence of alcohol, the oxidation of ethanolamine was presumed to proceed as follows.

$$NH_2CH_2CH_2OH \xrightarrow{O_2} NH_3 + \underset{\displaystyle CH_2OH}{\overset{\displaystyle CHO}{|}} + H_2O_2 \xrightarrow{\text{catalase}} \underset{\displaystyle CHO}{\overset{\displaystyle CHO}{|}} + 2H_2O + NH_3$$

Moreover, carbonyl reagents such as semicarbazide, hydrazide, hydroxylamine inhibited this reaction entirely (100%) at a concentration of $10^{-3}M$.

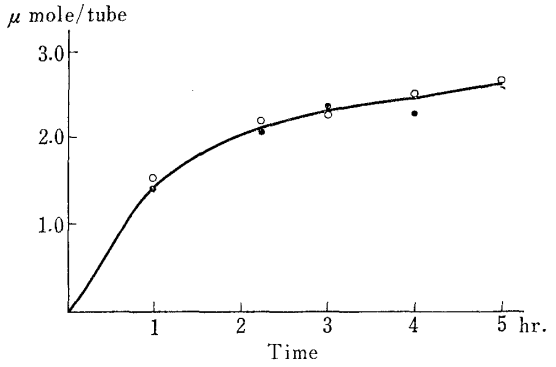

Fig. 5. Relationship between Oxygen Consumption and Ammonia Formation during the Course of Oxidation of Ethanolamine

Reaction mixture (2.0 ml.) was incubated in a Warburg vessel at the conditions described in text. At time intervals, the reaction was stopped and the amount of ammonia produced was estimated.

●—● oxygen consumption
○—○ ammonia formation

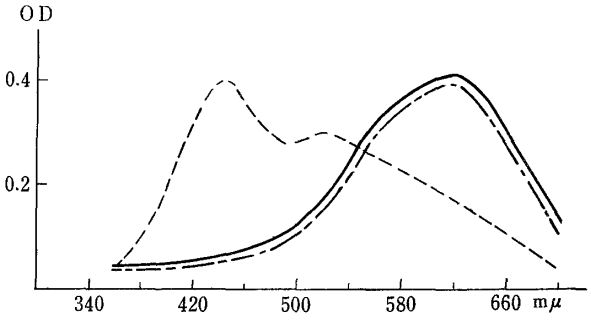

Fig. 6. Absorption Spectra of 2,4-Dinitrophenylhydrazone Derivatives

2,4-Dinitrophenylhydrazones derived from glycolaldehyde, glyoxal and from the oxidation product of ethanolamine were dissolved in 90% Me_2CO containing 0.1*N* NaOH and their absorption spectra were measured against reagent blank.

----- 2,4-dinitrophenylhydrazone of glycolaldehyde
——— 2,4-dinitrophenylhydrazone of glyoxal
----- 2,4-dinitrophenylhydrazone of oxidation product from ethanolamine

Separation of Oxidation Product

To 50 ml. reaction mixture after reaction at 37° for 2 hours, 10 ml. of 30% trichloroacetic acid was added to stop the reaction and to remove proteins. To the supernatant, an equal volume of *N*HCl solution saturated with 2,4-dinitrophenylhydrazine was added and the mixture was allowed to stand in refrigerator to give an amorphous precipitate. This precipitate was recrystallized from dioxane-ethanol (1:1) yielding a crystal of d.p 315°. Whereas bis-2,4-dinitrophenylhydrazone of glyoxal showed d.p 316°, 2,4-dinitrophenylhydrazone of glycolaldehyde was of m.p 155°. Judging from the absorption spectra of 2,4-dinitrophenylhydrazones derived from the oxidation products and that of glyoxal as well as glycolaldehyde dissolved in 90% acetone in 0.1 *N* sodium hydroxide (Fig. 6), perfect coincidence was found between derivatives from the oxidation product and glyoxal. The result of elemental analysis was as follow. *Anal.* Calcd. for $C_{14}H_{10}N_8O_8$: C, 40,19; H, 2.39; N, 26.79. Found : C, 40.26; H, 2.46; N, 26.09.

From these results, the crystal obtained from the reaction product was identified as bis-2,4-dinitrophenylhydrazone of glyoxal. No catalase was added to the reaction mixture in this case. Therefore, it was demonstrated that ethanolamine was oxidized to glyoxal in the absence of catalase.

Table I. Relative Rates of Reaction of Various Substrates by the Present Enzyme Preparation

	Relative rate		Relative rate
Ethanolamine	0.32	4-Hydroxybutylamine	0
N-Methylethanolamine[a)]	0	3-Hydroxy-4-aminobutyric acid	0
N,N-Dimethylethanolamine[b)]	0	Spermine	1.0
Choline[c)]	0	Spermidine	0.8
3-Hydroxypropylamine	0.08	Butylamine	0.72
2-Hydroxy-*n*-propylamine	0		

a) Methylamine was estimated by the method of Pesez, *et al.* (Bull. soc. chim. France, **1953**, 754),
b) Dimethylamine was estimated by the method of Stanley, *et al.* (Anal. Chem., **23**, 1779 (1951)).
c) Trimethylamine was estimated by the method of Hayashi, *et al.* (This Bulletin, **10**, 533 (1962)).
All the substrates except spermine and spermidine were used at the concentration of $5\times10^{-3}M$. Spermine and spermidine were used at $10^{-3}M$. Other experimental conditions are the same as described in text. Relative rates were calculated from the amount of NH_3 formed after incubation for 30 min.

Substrate Specificity

The substrate specificity of this partially purified preparation was shown in Table I. It has been stated formerly that this preparation oxidizes polyamines and butylamine. In addition to ethanolamine, 3-hydroxypropylamine was also oxidized by this enzyme.

Discussion

There have been several literatures on the degradation of ethanolamine. Weissbach and Sprinson[7] studied on the metabolism of ethanolamine in rat and pigeon using ethanolamine [1-^{13}C, D-2-^{14}C], or ethanolamine[1-^{13}C, 2-^{14}C], and deduced the formation of glycolaldehyde as an intermediate, but the direct evidence for the presence of glycolaldehyde was not given. Cohen, *et al.*[8] informed that Clostridium can hydrolyze ethanolamine into ethylene glycol and ammonia, and authors[9] have reported that *Proteus morganii* causes non-oxidative deamination of ethanolamine to give acetaldehyde and ammonia. As stated in present paper, ethanolamine was oxidized to glyoxal and ammonia by the enzyme in beef serum. And the ratio of oxygen consumption to ammonia formation was 1:1 in the presence or absence of catalase. When spermine was used as substrate in this system, the ratio of O_2: NH_3 was 2:1 in the absence of catalase, but 1:1 in the presence of catalase. While it was certified that the oxidation product is glyoxal, a question whether glycolaldehyde once formed is further oxidized to glyoxal or ethanolamine is oxidized concurrently with deamination to glyoxal could not be solved.

The amount of glyoxal formed during the course of reaction was followed by diphenylamine method of Dische, *et al.*,[10] which always corresponded to 50~70% of the amount of oxygen uptake or ammonia formation. This seemed to suggest that there may be another oxidation pathway other than the formation of glyoxal. Accordingly in expectation that glycolaldehyde once formed might be oxidized in a certain degree to glycolic acid, this was examined by Calkins 2,7-dihydroxynaphthalene method,[11] revealing the absence of glycolic acid in the oxidation mixture.

About purification of amine oxidase in beef serum, there is an excellent paper by Yamada and Yasunobu.[3] Yamada and Yasunobu resolved partially purified preparations of amine oxidase into two or three discrete, enzymatically active fractions by a procedure of DEAE-cellulose and hydroxylapatite chromatography, and obtained two crystalline enzymes individually from two fractions. However, whether the two crystalline enzymes are identical or not was not described. The preparation used in the present experiments was a partially purified one, and it could not be compared with Yamada's crystalline enzymes.

Although glyoxal was certified as an oxidation product of ethanolamine in this paper, further purification of enzyme may be necessary to demonstrate whether or not glycolaldehyde is an intermediate and glyoxal is a main product of oxidation of ethanolamine.

Summary

An enzyme oxidizing ethanolamine was partially purified from beef serum by fractionation with ammonium sulfate and DEAE-cellulose column chromatography. This partially purified preparation showed an optimum pH of 7.5 and apparent Km of $4\times 10^{-3}M$ for ethanolamine. Without regard to the addition of catalase, the ratio of oxygen consumption to ammonia formation was 1:1 and the oxidation product of ethanolamine was identified as glyoxal.

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