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9. Tsutomu Unemoto: Studies on Polyamines. III.*1 A New Spectrophotometric Method for the Estimation of Amine Oxidase Activity in Beef Serum.

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Previous methods for the estimation of amine oxidase activity in beef serum are based on the determination of oxygen consumption[1] and on a spectrophotometric assay of the change in absorption spectrum occurring when benzylamine, which serves as a substrate of this enzyme, is oxidized to benzaldehyde.[1] Although the latter method was found to be convenient in practice, it cannot be applied on the direct estimation of the activity of amine oxidase that oxidize polyamines, such as spermidine and spermine.

First, in an attempt to measure the activity of amine oxidase using polyamines as substrates, a fact that the oxidation product of polyamines, probably aminoaldehyde, forms a fluorescent compound with resorcinol*1,[2] was referred to. This method was indeed successful for the present purpose.[3] However, the method was inconvenient for the quantitative measurement of enzyme activity, since a reference standard was always required in every estimation. Therefore another method of estimation of the aldehydes derived from polyamines has been studied. The use of hydroxylamine, semicarbazide, thiosemicarbazide and 2,4-dinitrophenylhydrazine as aldehyde reagents were tested and, among them, thiosemicarbazide was found to be the most satisfactory for the present purpose.

After oxidation of spermidine and spermine with the amine oxidase, the oxidation mixture was allowed to react with $5\times10^{-3}M$ thiosemicarbazide in $0.1N$HCl. As shown in Fig. 1, absorption maximum of high intensity of the resultant thiosemicarbazones appeared at 264 mμ in both cases. Judging from the wave length of the absorption band, it is evident that the aldehydes produced by the enzymatic oxidation are simple aldehydes which do not contain conjugated double bonds in their molecules. The reaction of thiosemicarbazide with the resultant aldehydes was complete in 10 minutes at 37° as indicated in Fig. 2. The rate of increase in optical density at 264 mμ during the course of oxidation of spermidine or spermine agreed well with that of oxygen consumption (Fig. 3). Proportional relationship between the optical density of thiosemicarbazones and the concentration of enzyme preparation is also indicated in Fig. 4. From these results a new spectrophotometric method for the estimation of amine oxidase activity using spermidine and spermine as substrates was established as described in Experimental.

Although the structure of aldehydes produced from polyamines by enzymatic oxidation has not yet been identified, the author posturated in the previous paper*1 that 3-aminopropionaldehyde and 3-hydroxypropionaldehyde may be produced by the oxidation of spermidine and spermine. Therefore these aldehydes were synthesized and their thiosemicarbazones were examined in comparison with those obtained from the oxidation products of the polyamines. The derivatives of 3-aminopropionaldehyde and

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*1 Part II: This Bulletin, **11**, 1255 (1963).

*2 Okubo, Narashino, Chiba-ken (蔵本 力).

1) C.W. Tabor, H. Tabor, S.M. Rosenthal: J. Biol. Chem., **208**, 645 (1954).

2) T. Unemoto, K. Ikeda, M. Hayashi, K. Miyaki: This Bulletin, **11**, 148 (1963).

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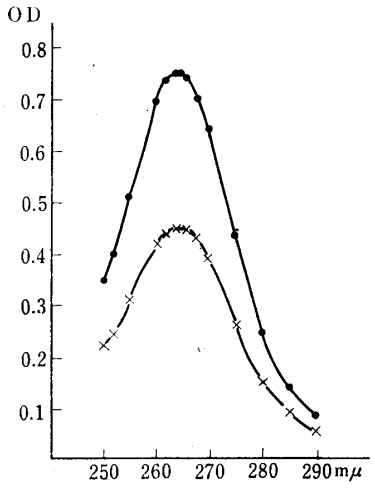


Fig. 1. Absorption Spectra of Thiosemicarbazones of Aldehydes

Reaction mixture contained spermidine ($5\times10^{-4}M$) or spermine ($4\times10^{-4}M$), 0.1M phosphate buffer (pH 7.0) and 1.65 mg./ml. of amine oxidase (see Experimental). After the complete oxidation of substrate at 37°, 0.5 ml. aliquot of the reaction mixture was mixed with an equal volume of $10^{-2}M$ thiosemicarbazide in 0.2N HCl and the mixture was further incubated for 10 min. at 37°. This was diluted with 9.0 ml. of H_2O and its absorption spectrum was measured against a blank which contained all of the components except substrate.

×—× Spermidine as a substrate (final concentration being $2.5\times10^{-5}M$)
•—• Spermine as a substrute (final concentration being $2.0\times10^{-5}M$)

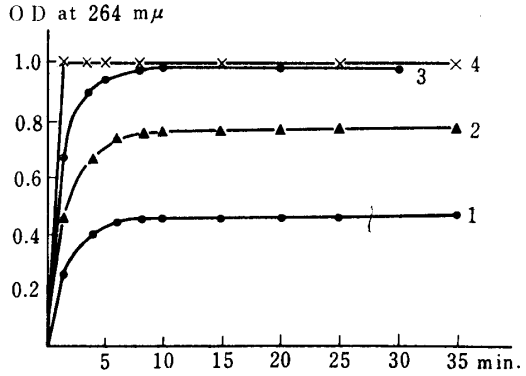


Fig. 2. Time Course of the Formation of Thiosemicarbazones of various Aldehyde Preparations

To an adequate amount of oxidation mixture (conditions are the same as in Fig. 1) or aldehyde solutions, an equal volume of $10^{-2}M$ thiosemicarbazide in 0.2N HCl was added and incubated at 37°. At intervals, 0.5 ml. of the mixture was taken and it was diluted with 4.5 ml. of H_2O. The formation of thiosemicarbazones was followed by measuring the optical density at 264 mμ against blank.

1: Oxidation products of spermidine($2.5\times10^{-5}M$)
2: Oxidation products of spermine ($2.0\times10^{-5}M$)
3: Aminopropionaldehyde ($5\times10^{-5}M$)
4: 3-Hydroxypropionaldehyde ($5\times10^{-5}M$)

Concentrations indicated in parentheses represent final concentration.

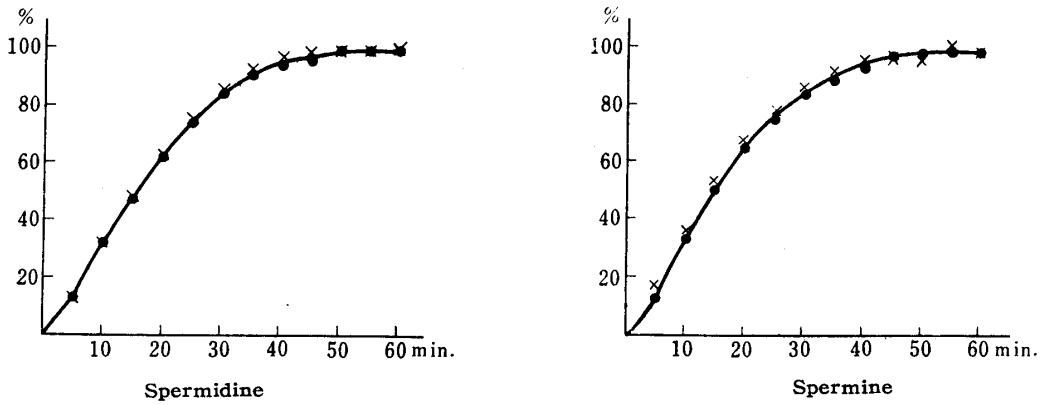


Fig. 3. Parallelism between Oxygen Consumption and Aldehyde Formation

Oxidation of polyamines was performed at the same conditions as described in Fig. 1. Oxygen consumption was followed by a conventional Warburg apparatus in a total volume of 4.0 ml. The increase in optical density at 264 mμ was measured by treating 0.5 ml. of the reaction mixture as described in Fig. 1. The oxygen consumption and the increase in optical density were compared as per cent of each maximum value.

3-hydroxypropionaldehyde showed the absorption band at 264 mμ and the molar extinction coefficients at this wave length were 1.96×10^4 and 2.04×10^4 liter · mole^{-1} · cm^{-1}, respectively. The molar extinction coefficients as calculated from Figs. 1 and 2, assuming that spermidine and spermine used as substrates were oxidized quantitatively into respective aldehydes, gave 1.92×10^4 and 3.75×10^4 liter · mole^{-1} · cm^{-1} for the thiosemicarbazones from spermidine and spermine, respectively. Since the thiosemicarbazones

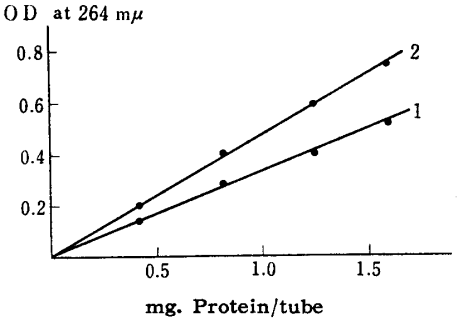


Fig. 4. Proportionality between Optical Density and Protein Concentration of Enzyme Preparation

Determination was made under the conditions described in the text.
1: Incubation period is 10 min.
2: 15 min.

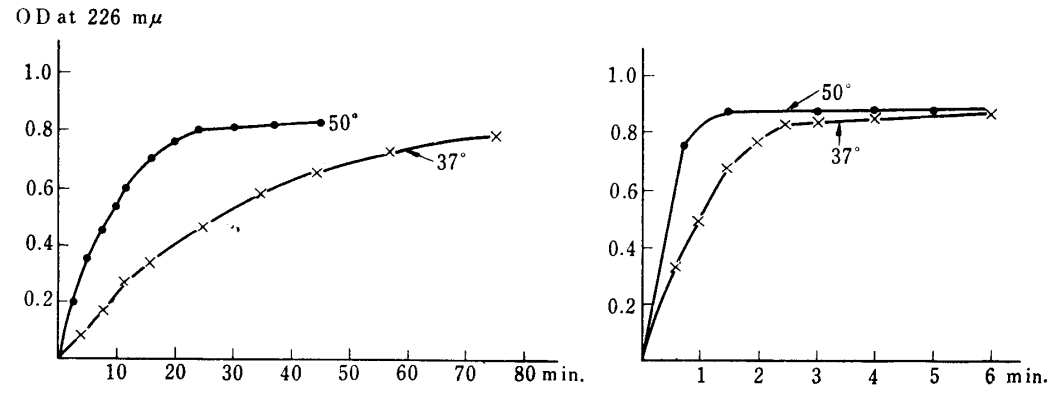
of simple aldehydes give ε values of around 2×10^4 liter · mole^{-1} · cm^{-1}, it is evident that one mole of aldehyde group was produced from one mole of spermidine and two moles from one mole of spermine by the enzymatic oxidation. Such observation bears out the reaction scheme of this enzyme presented in the previous report.*[1] These values facilitate the quantitative measurement of the oxidase activity as being expressed in terms of μ moles of spermidine or spermine oxidized per unit time.

Experimental

Materials——Spermidine and spermine were obtained from Nutritional Biochemicals Corp. Other reagents used in this experiment were commercial products (G. R.).

3-Aminopropionaldehyde Diethyl Acetal——It was synthesized from 3-chloropropionaldehyde diethyl acetal according to the following procedure. A mixture of 300 ml. of abs. EtOH and 15 g. of 3-chloropropionaldehyde diethyl acetal (synthesized from acrolein and dry HCl in EtOH; $b.p_{16}$ 70°) was cooled at −10° and saturated with dry NH_3 gas. It was allowed to react at 120° for 30 min. in an autoclave. After evaporation of the solvent under reduced pressure, the residue was crystallized twice from Et_2O to yield 10 g. of 3-aminopropionaldehyde diethyl acetal·HCl, which was very hygroscopic and its melting point could not be measured. *Anal.* Calcd. for $C_7H_{18}O_2NCl$: C, 45.77; H, 9.88; N, 7.63; Cl, 19.30. Found: C, 45.77; H, 9.86; N, 7.80; Cl, 18.97.

3-Hydroxypropionaldehyde Diethyl Acetal——Ten grams of 3-chloropropionaldehyde diethyl acetal was added to 300 ml. of 0.3*N* NaOH and hydrolyzed at 120° for 20 hr. with stirring. After the end of reaction, 100 g. of K_2CO_3 was dissolved and the mixture extracted three times with an equal volume of Et_2O. The extracts were combined, dehydrated with K_2CO_3 and Et_2O removed by evaporation. Then, the residue was fractionally distilled. Four grams of 3-hydroxypropionaldehyde diethylacetal ($b.p_{20}$ 98°) was obtained. *Anal.* Calcd. for $C_7H_{16}O_3$: C, 56.73; H, 10.88. Found: C, 56.80; H, 10.91.



3-Aminopropionaldehyde diethyl acetal 3-Hydroxypropionaldehyde diethyl acetal

Fig. 5. Hydrolysis of Acetals

Acetals ($10^{-4}M$) were hydrolyzed in 0.05*M* HCl containing $10^{-2}M$ semicarbazide at the temperature indicated in the Fig. At time intervals, the optical density of reaction mixture was measured at 226 mμ against blank. The rate of increase in the optical density corresponds to that of hydrolysis of acetals.

Hydrolysis of Acetals——The acetals synthesized as described above were hydrolyzed in 0.05*N* HCl containing $10^{-2}M$ semicarbazide, and the rate of hydrolysis was followed by measuring the increase in optical density at 226 mμ, an absorption maximum of semicarbazones of these aldehydes, as indicated in Fig. 5. The hydrolysis of 3-aminopropionaldehyde diethyl acetal was completed in 30 min. at 50°, whereas the hydrolysis of 3-hydroxypropanal diethyl acetal was much more rapid than the former and completed in 90 sec. at 50°. For the preparation of aldehyde solution, each acetal was incubated at 50° in 0.05*N* HCl for the period mentioned above.

Enzyme Preparation——The enzyme preparation was obtained according to the method described in the previous paper.[3] The specific activity of amine oxidase used in this experiment was 0.15 μmole of spermidine consumed per 10 min. and mg. of protein.

Enzyme Assay Method——To an adequate amount of enzyme solution, 0.1 ml. of 0.5*M* phosphate buffer (pH 7.0) was added and the total volume was made up to 0.4 ml. with H_2O. After equilibration of temperature at 37°, 0.1 ml. of 0.01*M* substrate (spermidine or spermine) was added and incubated for 10 min. At the end of incubation, 0.5 ml. of $10^{-2}M$ thiosemicarbazide dissolved in 0.2*N* HCl was added and the reaction mixture was further incubated for 10 min. at the same temperature. Then, it was diluted with 9.0 ml. of H_2O and the optical density was measured at 264 mμ against a blank which contained all of the reagents except substrate.

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Summary

A new spectrophotometric method for the estimation of amine oxidase activity in beef serum was established using spermidine or spermine as a substrate of the oxidase. This method is based on the measurement of optical density at 264 mμ of thiosemicarbazones of aldehydes derived from respective polyamines. From the values of molar extinction coefficients of the thiosemicarbazones, the amine oxidase activity can be expressed in terms of μmoles of spermidine or spermine oxidized per unit time and mg. protein. It was also found that one mole of spermidine and spermine were converted into one and two moles, respectively, of simple aldehyde by the enzymatic oxidation.

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