Chem. Pharm. Bull. 25(1) 185—191 (1977) UDC 577.159.02:547.556.8.09

A Spectrophotometric Assay of Histaminase Activity based on the Hydrazone Derivative of Imidazole Acetaldehyde with 2,4-Dinitrophenylhydrazine

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(Received January 24, 1976)

The activity of histaminase by using histamine as a substrate was spectrophotometrically estimated as the hydrazone derivative of imidazole acetaldehyde with 2,4-dinitrophenylhydrazine (DNP).

The measurement of enzyme activity in pig kidney was based on oxygen consumption in the presence of histamine by means of oxygen electrode, and the assay based on hydrazone derivative was performed with a spectrophotometer.

In determination of oxygen consumption, there was a remarkable difference in the patterns of activities under the conditions of incubation temperature of 38° and 60°. The maximum activity of histaminase at 60° was about 2.4 times higher than that at 38° when the concentration of histamine was 10^{-4}M.

By measuring the absorbance of the hydrazone derivative of imidazole acetaldehyde formed in the presence of 3×10^{-4}M histamine at 60°, it could be compared with oxygen consumption proportional to an increased amount of enzyme. Isooctane extractable hydrazones and unreacted DNP disturbed markedly the spectrophotometric assay, but these substances could be removed by use of a mixed solvent of isooctane and $CHCl_3$.

If aldehyde compound is formed over 1 μM/min or if oxygen is consumed over 15 μM/min even in the incubation temperature of 38°, it is indicated that hydrazone derivative of aldehyde compound can be spectrophotometrically detected and that the assay method can be applied to the measurement of histaminase activity.

Keywords——histaminase; 2,4-dinitrophenylhydrazine; histamine; pig kidney; isooctane; oxygen electrode; spectrophotometer; imidazole acetaldehyde; oxidative deamination

Introduction

Although there is no apparently discriminative definition between two terms of histaminase and diamine oxidase (diamine: oxygen oxidoreductase [deaminating], EC 1.4.3.6) (DAO), the former name has been conventionally utilized when histamine was used as a substrate. In measuring the activity of an enzyme, a radiometric assay method with a labelled substrate is recognized as a highly reliable method. In the case of the estimation of histaminase activity there are two kinds of assays, that is, by the disappearance of ring-labelled ^{14}C-histamine[2)] and by the release of tritiated water produced from side chain labelled ^{3}H-histamine[3)] as substrates. These assays are quantitative but not directly related to the mechanism of formation of aldehyde. Parts of labelled-histamine appear to be incorporated into the enzyme protein.[4)] In order to overcome this handicap, an attempt has been made to measure the radioactivity of the product probably obtained by reacting ring-labelled histamine metabolite with aminooxyacetic acid.[5)] There are, besides, benzylamine and its relates, good substrates of DAO, used for spectrophotometric methods by measuring directly

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2) H.G. Roscoe and D. Kupfer, *Anal. Biochem.*, **47**, 418 (1972).
3) M.A. Beaven and S. Jacobsen, *J. Pharmacol. Exptl. Therap.*, **176**, 52 (1971).
4) H. Kumagai, T. Nagate, H. Yamada, and H. Fukami, *Biochim. Biophys. Acta*, **185**, 242 (1969).
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the aldehyde compounds produced from them,[6] and aldehyde compounds for this procedure are limited to a conjugate molecule. However, aldehydes produced from other substrates and their analogues[7] instead of these substrates have non-conjugated substituents and mostly a low molar extinction coefficient in the ultraviolet region. This is simply solved by a procedure having a bathochromic and hyperchromic effects on these aldehydes. In spectrophotometric methods in such a secondary manner, there are few assays based on the reactions of γ-aminobutyraldehyde from putrescine with *o*-aminobenzaldehyde,[8] of 4-dimethylaminomethylbenzaldehyde and 4-nitrobenzaldehyde from their amines with 4-nitrophenylhydrazine,[9] and of 3-aminopropionaldehyde from spermidine and 3-hydroxypropionaldehyde from spermine with thiosemicarbazide.[10]

In 1959 Kapeller-Adler and Fletcher identified that imidazole acetaldehyde was formed by the action of histaminase on histamine.[11] Direct determination of this aldehyde compound produced is the most suitable in various assay methods employed for estimating histaminase activity. However, it is not easy to measure histaminase activity based on aldehyde compound in an enzymic reaction with histamine substrate because imidazole acetaldehyde itself has a low molar extinction coefficient and a very hygroscopic property. In addition, as considerably important factors, there are some interferences from autoxidation by the enzyme, the occurence of endogenous ammonia, and the possible coexistence of xanthine oxidase or aldehyde oxidase related to the alteration of imidazole acetaldehyde into imidazole acetic acid,[12] especially in the crude enzyme preparations. Therefore, in the estimation of histaminase activity by using histamine as a substrate it is desirable to measure the imidazole acetaldehyde produced as its derivative with carbonyl reagents, in which 2,4-dinitrophenylhydrazine (DNP) shifts the absorption maximum of the Schiff base to a near-ultraviolet region.

On the basis of such a conception, we designed an experimental system for the assay of histaminase activity being dependent on an enzymatic product, imidazole acetaldehyde. In the present study, an attempt was first made to increase oxygen uptake by raising the incubation temperature by means of oxygen electrode, in order to produce a detectable amount of aldehyde compound, according to a stoichiometric reaction. Subsequently, a research was spectrophotometrically made to elucidate the relation of the aldehyde formation and the hydrazone derivative produced with 2,4-dinitrophenylhydrazine.

Experimental

The crude enzyme of histaminase was prepared from pig kidney according to the method described by Suetsugu[13] and the supernatant of $9000 \times g$ was used as an enzyme material. The ratio of optical density at 280 nm to that at 260 nm was 0.903. The activity of histaminase by using histamine as a substrate was determined by measuring oxygen consumption by means of oxygen electrode.[14] Hydrazone derivative was determined by measuring its absorbance at 360 nm with a spectrophotometer.

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The Assay of Histaminase Activity based on the Hydrazone Derivative of Imidazole Acetaldehyde——Enzymatic imidazole acetaldehyde formed by the oxidative deamination of histamine was estimated as its hydrazone derivative produced with DNP. The crude enzyme of histaminase was added to 0.1 M sodium phosphate buffer (pH 7.1) and the suspension was then preincubated at 60° for 10 min. After adding a final concentration of 3×10^{-4} M histamine the total volume of 12 ml was incubated at 60° for 5 min. To this incubation solution was added a solution of 0.5 mM DNP—alcohol (ketone-free ethyl alcohol) adjusted to pH 1.5 with 40% H_3PO_4 to give a total volume of 20 ml (the pH of the reaction mixture was 2.5). The reaction mixture was then shaken at 60° for 30 min. After centrifuging at $2000\times g$ for 5 min, an appropriate volume of the supernatant was shaken with two volumes of the mixed solvent of 2,2,4-trimethylpentane (isooctane)–$CHCl_3$ (1:1) to remove unreacted DNP and isooctane extractable hydrazones. The concentration of the hydrazone derivative of imidazole acetaldehyde present in H_2O layer was estimated by measuring the absorbance at 360 nm.

Syntheses of Imidazole Acetaldehyde from *l*-Histidine and NaClO, and of Its Derivative with DNP[11,15]——The hydrazone derivative of imidazole acetaldehyde was produced from the chemical reaction of DNP with imidazole acetaldehyde synthesized from *l*-histidine and NaClO.

The calculated volume of NaClO was added dropwise to a solution of 15 mmoles of *l*-histidine dissolved in 0.9 ml of conc.HCl and 45 ml of H_2O at 0°, and then after adding 0.5 ml of conc.HCl the solution was allowed to stand at 0° for 10 min. The pale green solution obtained was concentrated to dryness under reduced pressure. To this residue was added an appropriate volume of ketone-free alcohol containing a few drops of conc.HCl to remove undissolved NaCl, and the filtrate was again evaporated to dryness (the crude imidazole acetaldehyde was precipitated from the clear alcohol solution by adding ether: $\varepsilon=4550$ at 210 nm; $\varepsilon=920$ at 250 nm, in H_2O). This residue being made to react with DNP, 2,4-dinitrophenylhydrazone of imidazole acetaldehyde was derived and recrystallized from alcohol: reddish yellow needles, mp 124—124.5° (lit.[11] 124—126°); $\varepsilon=2.35\times10^4$ at 225 nm; $\varepsilon=1.55\times10^4$ at 250 nm; $\varepsilon=3.15\times10^4$ at 360 nm, in EtOH.

Results and Discussion

The Effect of Incubation Temperature on Histaminase Activity Based on Oxygen Consumption and Conditions to Its Assay Based on Hydrazone Derivative

In order to design an experimental system for the assay of histaminase activity based on hydrazone derivative, the effect of incubation temperature on histaminase activity based on oxygen consumption was observed by means of oxygen electrode. In both conditions of incubation temperature of 38° and 60°, the patterns of histaminase activities were markedly different from each other and a moderately high temperature of 60° caused an increase in the activity with the crude enzyme of histaminase (Fig. 1). When the concentration of histamine was 10^{-4}M, oxygen uptake at 60° was about 2.4 times higher than that at 38° in 1 min. Oxygen uptake at 38° had a tendency to increase up to 5 min whereas that at 60° to decrease after 2 min in the presence of 10^{-4}M histamine (Fig. 2a and 2b). When the time-courses of oxygen consumption were followed by increasing the amount of enzyme under the conditions of 10^{-4}M histamine at 60° (Fig. 2c), the oxygen uptakes increased linearly with increasing amounts of enzyme within 1 min but they were not dependent on those after 2 min. At 60° oxygens were linearly consumed up to 3 min at 3×10^{-4}M and 5 min at 10^{-3}M histamine (Fig. 2b). Consequently, it is indicated that a concentration of 10^{-4}M of histamine which brought about the maximum activity (Fig. 1) is not an optimum condition in an enzymic reaction of 1 min or more at 60°.

These results obtained by means of oxygen electrode indicated that the conditions such as incubation temperature of 60°, incubation time of 5 min, substrate concentration of 3×10^{-4}M and enzyme amount of 1.5 ml, were preferably necessary to detect effectively a maximum amount of imidazole acetaldehyde produced by the oxidative deamination of histamine with histaminase. In determining this reaction time, substrate was supposed to be completely oxidized to aldehyde without subsequent oxidation by other enzyme.[12] That is, the time taken to degrade oxidatively 3×10^{-4}M of the substrate was calculated at 5 min from the oxygen uptake of 56 μM/min (Fig. 2b).

15) H. Tabor, "Methods in Enzymology," Vol. III, ed. by S.P. Colowick, and N.O. Kaplan, Academic Press Inc., New York, 1957, p. 623.

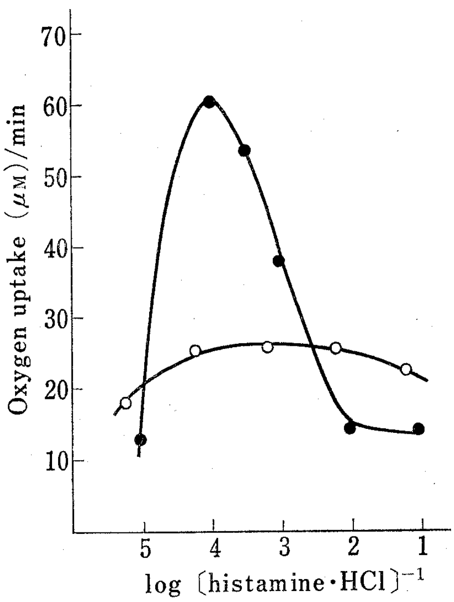

Fig. 1. Oxygen Uptake to Various Concentrations of Histamine under Oxidative Deamination by Histaminase

Oxygen consumption was measured by means of oxygen electrode. A solution containing 1.5 ml of the enzyme and 0.1M sodium phosphate buffer (pH 7.1) was preincubated at 38° (—○—) and 60° (—●—). After equilibrium was reached, various concentrations of histamine were added. The reactions were followed over 1 min. The reaction mixture was a total amount of 3.0 ml.

Spectrophotometric Separation of the Hydrazone Derivative of Imidazole Acetaldehyde

In spectrophotometric determination by the absorbance of hydrazone derivative, particularly unreacted DNP disturbed the assay because it has almost the same molar extinction coefficient with 2,4-dinitrophenylhydrazone of aldehyde compound. Therefore, the unreacted DNP was removed by an extraction procedure. After adding a solution of alcohol containing DNP at the end of incubation, the reaction mixture was shaken under an acidic condition with H_3PO_4 at 60° for 30 min and then the protein precipitated was removed by centrifugation. The supernatant was washed with isooctane and then with $CHCl_3$. Absorption spectra of each layer of isooctane (a), $CHCl_3$ (b), and H_2O (c) are shown in Fig. 3. A similar procedure was taken for an incubation solution without a substrate. The absorbance of isooctane layer increased with increasing amount of enzyme, but such spectra expected as the enzymatic product were not observed from H_2O layer (Fig. 4).

From the results mentioned above and the absorption spectrum of the hydrazone derivative of synthetic imidazole acetaldehyde (d on Fig. 3), it is considered that hydrazone derivative of enzymatic imidazole acetaldehyde is present in H_2O layer.

Subsequently, an attempt was made on the simplification of an extraction procedure. Namely, substances present in both layers of isooctane and $CHCl_3$ were simultaneously extracted by washing with a mixture of their solvents for the supernatant obtained after removal of protein. From the balance sheet that both contents of substances present in organic and

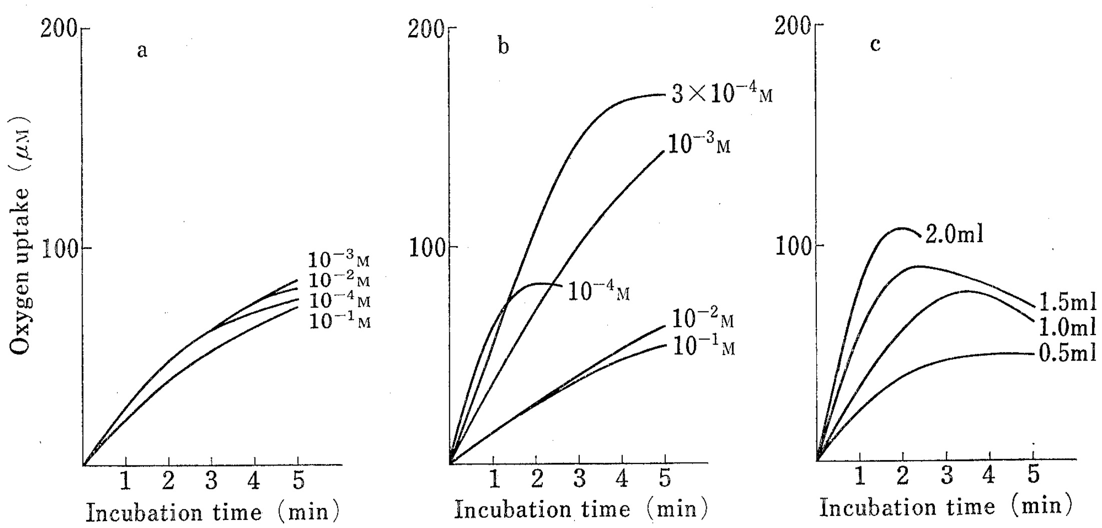

Fig. 2. Time-Course of Histaminase Activity

The changes of histaminase activities were followed over 5 min by means of oxygen electrode. After 1.5 ml of the enzyme in 0.1M sodium phosphate buffer (pH 7.1) were preincubated at 38° (a) and 60° (b) for 10 min, oxygen uptake was determined by adding various concentrations of histamine. The time-courses of histaminase activities under various amounts of the enzyme were followed after the addition of 10^{-4}M histamine at 60° (c). The reaction mixture was a total amount of 3.0 ml.

H_2O layers were expressed in the amount of DNP as shown in Table I, it was found that increasing amount of hydrazone derivative in H_2O layer corresponded to decreasing amount of DNP.

According to this procedure, the time-course of the formation of imidazole acetaldehyde was followed. Measured on the basis of the hydrazone derivative of imidazole acetaldehyde, the absorbances increased linearly over few minutes in concentrations of 3×10^{-4}M and 10^{-3}M

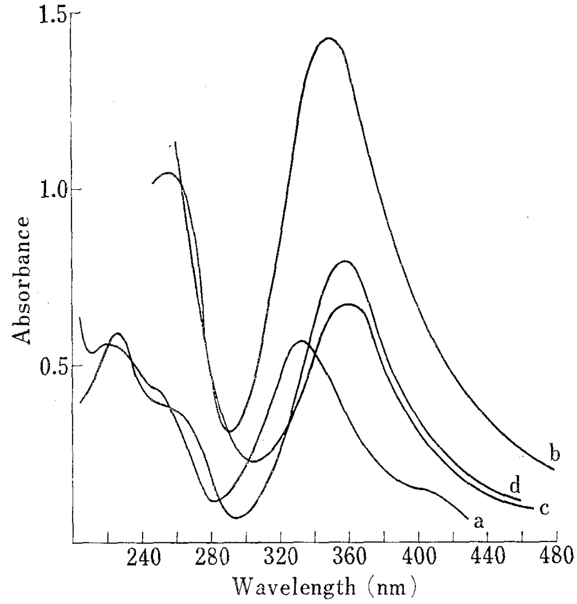

Fig. 3. Absorption Spectra of Two Hydrazone Derivatives of Synthetic and Enzymatic Imidazole Acetaldehyde, Isooctane Extractable Hydrazones, and Unreacted DNP

The hydrazone derivative of enzymatic imidazole acetaldehyde, isooctane extractable hydrazones, and unreacted DNP were separated from the reaction mixture. The enzyme amount of 1.8 ml and a final concentration of 3×10^{-4}M histamine were used. The mixture was incubated and DNP was added in the same manner as described in the text. After centrifugation the supernatant was washed with an equal volume of isooctane and then with $CHCl_3$. Spectra of (a), (b), (c), and (d) show hydrazone derivative in isooctane layer, unreacted DNP in $CHCl_3$ layer, the hydrazone derivative of enzymatic imidazole acetaldehyde in H_2O layer, and the hydrazone derivative of synthetic imidazole acetaldehyde of 25 μM in alcohol, respectively. Correction by enzyme as a blank was not made on the absorbance of (c).

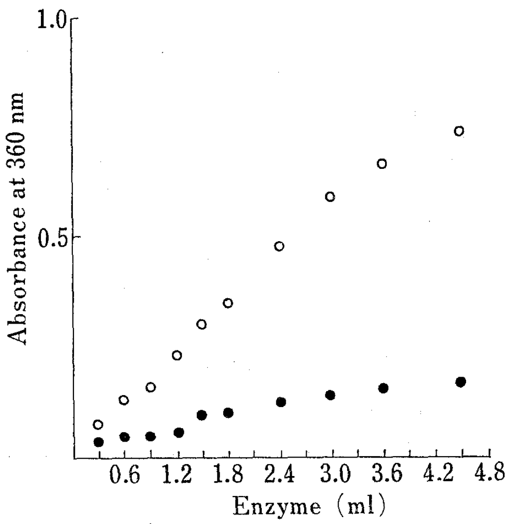

Fig. 4. Distribution of Isooctane Extractable Hydrazones

Various amounts of the enzyme were incubated in 0.1M sodium phosphate buffer (pH 7.1) at 60° for 5 min. Phosphate buffer was incubated as a blank. After adding DNP, the mixture was shaken at 60° for 30 min, as described in the text. After centrifugation the supernatant was washed with an equal volume of isooctane and then with $CHCl_3$. The absorbances of organic and H_2O layers were measured at 360 nm.

(○): isooctane layer

(●): H_2O layer

Table I. Balance Sheet (μM) of the Concentration of 2,4-Dinitrophenylhydrazine ($\varepsilon=1.21\times10^4$) at 360 nm[a)]

Histamine	Solvents	Enzyme (ml) 0	0.4	0.8	1.2	1.6	2.0	2.4
3×10^{-4}M	isooctane–$CHCl_3$	171	160	146	134	125	116	105
	H_2O	28	40	54	65	75	84	94
—	isooctane–$CHCl_3$	171	169	164	163	162	161	161
	H_2O	30[b)]	31	35	37	38	39	38

a) Each concentration was calculated on the basis of Lambert-Beer's law and ε of DNP was determined in a mixed solvent of isooctane and $CHCl_3$ (1:1).
b) The absorbance of DNP slightly remaining in H_2O layer.

histamine, in which the linearities were throughout 7 min and 10 min (Fig. 5). Further experiment should be carried out to obtain information as for the change of aldehyde formation over 10 min.

As mentioned above, histaminase activity determined as a function of the hydrazone derivative of imidazole acetaldehyde was able to be compared with oxygen consumption proportional to an increased amount of enzyme, when the concentration of histamine was 3×10^{-4}M (Fig. 6).

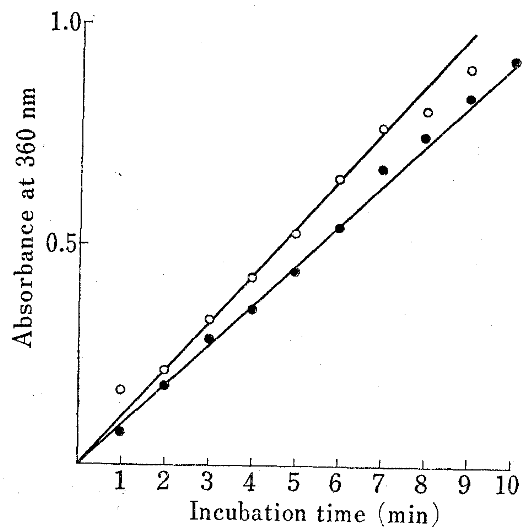

Fig. 5. Time-Course of Histaminase Activity Based on Hydrazone Derivative

The enzyme of 1.5 ml in 0.1M sodium phosphate buffer (pH 7.1) was incubated at 60° from 1 to 10 min after the addition of histamine of 3×10^{-4}M (—○—) or 10^{-3}M (—●—). A phosphate buffer solution containing enzyme was incubated as a blank. After reacting with DNP, the hydrazone derivative of imidazole acetaldehyde was separated from the reaction mixture by once washing with two volumes of isooctane–$CHCl_3$ (1:1) and histaminase activity was estimated by measuring the absorbance of H_2O layer at 360 nm. Details are described in the text.

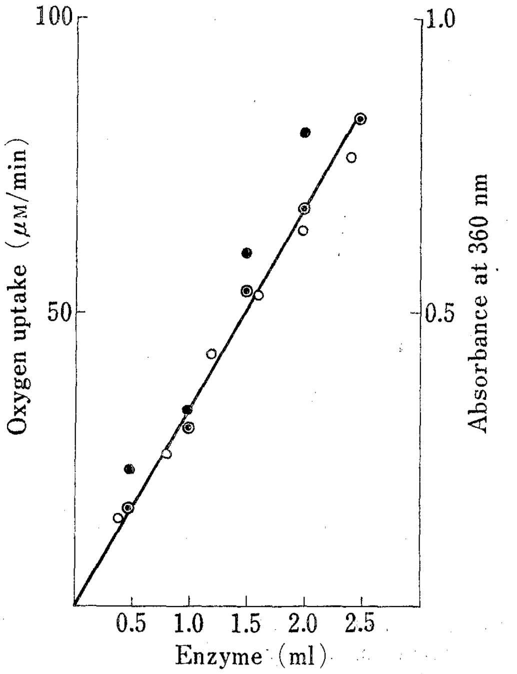

Fig. 6. Correlations between Oxygen Consumption and Aldehyde Formation

The consumption of oxygen and the formation of imidazole acetaldehyde were determined by measuring oxygen uptake by means of oxygen electrode for 1 min and the absorbance of the hydrazone derivative formed with DNP 5 min after incubation, respectively. The histamine concentrations of 10^{-4}M (—●—) and 3×10^{-4}M (—⊙—) were used in the former measurement and 3×10^{-4}M (—○—) in the latter one. Details are described in the text. Oxygen uptake is the value obtained by adding a substrate after equilibrium was reached and the absorbance is determined by subtraction of that obtained from a buffer solution containing enzyme as a blank.

After synthesizing imidazole acetaldehyde from the reaction of *l*-histidine with a calculated amount of NaClO, its hydrazone compound was derived from DNP. The calibration curve of 2,4-dinitrophenylhydrazone of imidazole acetaldehyde, as seen in Fig. 7, could be assigned to oxygen uptakes.

Compared aldehyde formation with oxygen consumption, it was found that oxygen uptake was 53 μM/min whereas the hydrazone product amounted to about 3.3 μM/min, when a concentration of 3×10^{-4}M histamine was oxidatively deaminated by the enzyme amount of 1.5 ml. In the oxidative deamination of tyramine substrate by monoamine oxidase (monoamine: EC 1.4.3.4) (MAO) as reported previously,[16a)] the enzymic reaction of 1 min-period was

16) *a)* K. Kamijo and T. Watanabe, *Chem. Pharm. Bull.* (Tokyo), **24**, 698 (1976); *b)* K. Kamijo, S. Sho, and T. Egashira, *Jap. J. Cli. Path.*, **21**, 137 (1973).

stoichiometric whereas that of 60 min-period resulted in the consumption of oxygen exceeding the theoretical value. As a method similar to our procedure, there is an assay method, in which the activity of partially purified amine oxidase in bovine serum was estimated on the basis of thiosemicarbazone of the aldehyde compound produced from polyamine as a substrate.[10] It was demonstrated that there was parallelism between oxygen consumption and aldehyde formation by the respective uses of a Warburg apparatus and a spectrophotometer. On the contrary, we could not obtain a consistent result from both reaction systems by means of oxygen electrode and spectrophotometry. Regarding our inconsistent result, it is reasonable to consider that our enzyme is impure and, similarly to the behaviors of MAO as described in detail by Kamijo, *et al.*,[16] the property of histaminase itself may be multiple, as a result of oxidative deaminations of putrescine, cadaverine, and benzylamine by it.[7,13,17] In addition, the yields of hydrazone derivatives of carbonyl group may be lower compared with the result reported elsewhere.[9,18] But the condition to form hydrazone derivatives is chemically not vigorous and the result is rather good. Therefore, it should be demonstrated that the hydrazone product is obtained in a yield close to a theoretical value. In addition to the present quantitative assay, it is preferable to identify chemically 2,4-dinitrophenylhydrazone of enzymatic imidazole acetaldehyde after its isolation, as performed by Kapeller-Adler and Fletcher.[11]

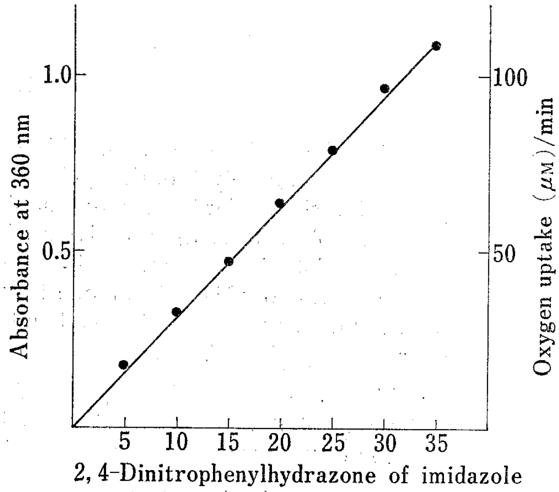

Fig. 7. Calibration Curve of 2,4-Dinitrophenylhydrazone of Imidazole Acetaldehyde and Its Comparison with Oxygen Uptakes

The absorbance of the hydrazone derivative of synthetic imidazole acetaldehyde was measured in alcohol at 360 nm. Syntheses of imidazole acetaldehyde and its hydrazone derivative are described in the text. The absorbances correspond to the value determined 5 min after incubation and their 100-fold values exhibit oxygen uptake of μM consumed per 1 min.

It is known that a moderately high temperature increases DAO activity[19] whereas it reduces MAO activity.[20] But Suetsugu reported that inactivation of about 60% was observed in DAO activity at 65°.[13] The present result shows that the aldehyde produced is detected as its hydrazone derivative, irrespective of a high or a low temperature, if the amount of the aldehyde produced by the degradation of a substrate under a physiological condition is enough to react with DNP (over 1 μM/min), that is, oxygen uptake measured by means of oxygen electrode is over 15 μM/min even at 38°. In conclusion, it is indicated that the assay method can be applied to the measurement of activity of an enzyme participating in the mechanism of oxidative deamination as well as to the assay of aldehyde dehydrogenase.[18]

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