Abstract—DAO was partially purified from hog kidney by a procedure involving differential centrifugation, heat treatment and ammonium sulfate fractionation. The enzymic properties of partially purified DAO were studied with cadaverine and histamine as substrates and the following results were obtained. The specific activities of hog kidney DAO at the final step of purification were 183-fold and 125-fold those of the crude homogenate with histamine and cadaverine, respectively, as substrates. With cadaverine and histamine the pS maxima values were $3 \times 10^{-3}$ M and $1 \times 10^{-2}$ M and the pH optima were 7.1 and 6.5, respectively. All the diamines tested, (i.e. cadaverine, putrescine, hexamethylenediamine, histamine, ethylenediamine and agmatine) were oxidized with this enzyme, while monoamine, (i.e. tyramine, benzylamine, serotonin, β-phenylethylamine, n-amyamine and n-propylamine) were not. DAO activity was inhibited by aminoguanidine, hydroxylamine, MPH and semicarbazide. The inhibitory effects were similar when either cadaverine or histamine was used as substrate. SH-reagents, such as PCMB and iodoacetamide, did not affect DAO activity, but various metal ions, such as CuCl, Cu(NO$_3$)$_2$, Zn(NO$_3$)$_2$, AICl$_3$ and Fe(NO$_3$)$_3$ were inhibitory. The inhibitory effect of AgNO$_3$ on DAO activity differed greatly, when using cadaverine and histamine as substrates. The temperature-inactivation curves of DAO measured with cadaverine and with histamine were different.

There have been reports on diamine oxidase (DAO) (E.C. 1.4.3.6. diamine: oxygen oxidoreductase (deaminating)), which catalyzes the oxidation of cadaverine, putrescine and agmatine, and histaminase which catalyzes the oxidation of histamine. Since the substrate specificities of DAO and histaminase are very similar (1–6), Zeller called both enzymes DAO. Goryachenkova (7) and Leloir (8) also suggested that DAO and histaminase were the same enzyme because they could not be separated by electrophoresis. Mondovi et al. (9) also stated that these two enzymes were identical since during purification the ratio of the activities in oxidation of cadaverine and histamine remained almost the same. On the other hand, Kapeller-Adler (10–11) reported that DAO and histaminase were different enzymes, since her histaminase preparation purified from hog kidney oxidized histamine but not cadaverine.

Thus although there have been many investigations on the possible identity of these two enzymes, the problem has yet to be clarified. In the present work the amine oxidase was partially purified from hog kidney, and the properties were studied using cadaverine and histamine as substrates.
MATERIALS AND METHODS

Measurement of DAO activity

Enzymic activity was estimated by measuring (12) oxygen consumption with a Clark's electrode. Reaction mixture in a volume of 2.9 ml containing enzyme solution and 0.033 M phosphate buffer, pH 7.1, was equilibrated at 38°C. The reaction was then started by adding 0.1 ml of substrate solution and oxygen consumption during the first min was measured. Enzymic activity was expressed as oxygen consumption (μM) per min per mg protein. Values were corrected by subtracting the value for non-enzymic oxidation of the substrate and for oxygen uptake by the enzyme with endogenous substrates without addition of exogenous substrate.

Measurement of MAO activity

Monoamine oxidase (MAO) activity was determined by the measurement of oxygen consumption (μM) at 38°C using a Clark's electrode as described by Kinemuchi (13).

Protein determination

The protein concentration was determined spectrophotometrically by measuring the absorbance at 280 nm.

RESULTS

DAO activity in hog kidney

Fresh hog kidney was cut into small pieces and homogenized with 3 volumes of 0.25 M sucrose solution containing 0.01 M phosphate buffer, pH 7.1 in a Waring blender and a Potter-Elvehjem glass homogenizer (F 1). The homogenate was centrifuged twice at 600 × g for 20 min. The resulting precipitate was dissolved in 0.1 M phosphate buffer, pH 7.1 and kept in an ice bath (F 2). The supernatant was centrifuged twice at 8,500 × g for 30 min. The resulting precipitate was dissolved in the same buffer (F 3). The supernatant was recentrifuged at 20,000 × g for 30 min and the precipitate was dissolved in the same buffer (F 4). The supernatant was centrifuged at 105,000 × g for 60 min. The precipitate was dissolved in the same buffer (F 5), and the final supernatant fraction (F 6) was obtained. The procedure of subcellular fractionation of the hog kidney homogenate is shown in Fig. 1.

The activities of DAO and MAO in each fraction were measured using cadaverine, histamine and tyramine as substrates and the data obtained are summarized in Table 1. When cadaverine and histamine were used as substrates, DAO activity of the supernatant obtained by centrifugation at 105,000 × g for 60 min (F 6) was about 50% of the activity of the homogenate (control) and the percentage activities in other fractions were less than 20% those shown in Fig. 1. Fractionation of hog kidney homogenate by differential centrifugation.
DIAMINE OXIDASE FROM HOG KIDNEY

TABLE 1. DAO and MAO activities in fractions during purification. Concentration of substrates was $3 \times 10^{-3}$ M.

| F | Centrifugation | Total volume (ml) | Relative activity (%) | Cadaverine | Histamine | Tyramine |
|---|----------------|-------------------|-----------------------|------------|-----------|----------|
| 1 | Homogenate     | 100               | 100                   | 100        | 100       | 100      |
| 2 | 0~600 x g (ppt.) | 60                | 17.0                  | 19.5       | 14.1      |
| 3 | 6000 x g~8,500 x g (ppt.) | 40            | 12.2                  | 10.0       | 57.0      |
| 4 | 8,500 x g~20,000 x g (ppt.) | 15            | 6.6                   | 8.5        | 14.3      |
| 5 | 20,000 x g~105,000 x g (ppt.) | 10            | 7.1                   | 10.5       | 2.8       |
| 6 | 20,000 x g~105,000 (sup.)   | 60            | 50.2                  | 51.0       | 2.4       |

of the control. The activity ratios for oxidation of cadaverine and histamine were similar in each fraction. With tyramine as substrate, MAO activity of the mitochondrial fraction (F 3) was found to be 57% that of the homogenate and the percentage activities in other fractions were less than 15% that of the control.

**Purification of hog kidney DAO**

The procedure for purification of hog kidney DAO is shown in Fig. 2. The homogenate was centrifuged twice at 10,000 $x$ g for 20 min and the precipitate was discarded. The supernatant was centrifuged at 105,000 $x$ g for 60 min. The supernatant thus obtained was heated at 60°C for 40 min and then centrifuged twice at 10,000 $x$ g for 10 min. Saturated ammonium sulfate solution in 0.1 M phosphate buffer was added drop-wise to the supernatant with stirring to yield a 50 to 60% saturation, after which the mixture was centrifuged at 10,000 $x$ g for 10 min. The precipitate was dissolved in a minimum amount of 0.1 M phosphate buffer. The solution was dialyzed overnight in a cold room against 0.01 M phosphate buffer, pH 7.1. DAO activity was measured with cadaverine and histamine as substrates. The yellowish brown enzyme preparation could be kept at -20°C for 2 months without loss of activity.

**Purity of fractions obtained during purification of DAO**

The DAO activities of the fractions obtained during purification were measured with cadaverine and histamine as substrates and are shown in Table 2. At the final step the activities with cadaverine and histamine, respectively, were 183-fold and 125-fold those of the homogenate.
The ratios of the activities with cadaverine and histamine at each step are also summarized in Table 2. The ratio was 1.46 at the final step but 0.82–1.08 at other steps. That is, the ratio of the activities with these two substrates only differed at the final step.

The values for recovery of activity at the final step measured with cadaverine and histamine, respectively, were approx. 30.5% and 21.1% of the total activity of the homogenate.

**pS Maxima and pH optima**

The pS maxima in the oxidation of cadaverine and histamine by the enzyme, were measured at substrate concentrations of $1 \times 10^{-5}$ M to $1 \times 10^{-1}$ M. As shown in Fig. 3

![Fig. 3. Activity-pS and pH curves.](image)

**TABLE 2. Activity and yield of partially purified DAO at each purification step.**

| Purification steps | Activity (µM/min./ml) | Specific act. $\times 10^{10}$ (Act./mg prot.) | Purity | P.Cad. | Yield (%) |
|--------------------|-----------------------|-----------------------------------------------|--------|--------|-----------|
|                    | Cad. | Hist. | Cad. | Hist. | Cad. | Hist. | P.Hist. | Cad. | Hist. |
| Homogenate         | 14.4 | 6.8   | 0.42 | 0.20  | 1.0  | 1.0   | 1.0     | 100  | 100   |
| 10,000×g sup.      | 11.7 | 5.4   | 1.58 | 0.72  | 3.7  | 3.4   | 1.08    | 58.0 | 56.1  |
| 105,000×g sup.     | 10.9 | 5.0   | 2.53 | 1.16  | 6.0  | 5.8   | 1.03    | 49.3 | 50.1  |
| Heat treatment sup.| 10.6 | 6.1   | 17.3 | 10.0  | 41.1 | 50.0  | 0.82    | -    | -     |
| Satur. fraction    | 91.7 | 30.0  | 80.6 | 25.1  | 183  | 125   | 1.46    | 30.5 | 21.1  |

The ratios of the activities with cadaverine and histamine at each step were also summarized in Table 2. The ratio was 1.46 at the final step but 0.82–1.08 at other steps. That is, the ratio of the activities with these two substrates only differed at the final step.

The values for recovery of activity at the final step measured with cadaverine and histamine, respectively, were approx. 30.5% and 21.1% of the total activity of the homogenate.

**pS Maxima and pH optima**

The pS maxima in the oxidation of cadaverine and histamine by the enzyme, were measured at substrate concentrations of $1 \times 10^{-5}$ M to $1 \times 10^{-1}$ M. As shown in Fig. 3.
the activity-pS curves with these substrates had a typical bell shape. The pS maximum was found to be $3 \times 10^{-5}$ M with cadaverine and $1 \times 10^{-3}$ M with histamine. The rates of oxidation of $3 \times 10^{-5}$ M cadaverine and $1 \times 10^{-3}$ M histamine at various pH values were investigated using phosphate buffer (0.1 M KH$_2$PO$_4$—0.1 M Na$_2$HPO$_4$) from pH 5.5 to 7.0 and Tris-HCl buffer from pH 7.0 to 8.5. As shown in Fig. 3 the pH optima were slightly different that is, pH 7.1 with cadaverine and pH 6.5 with histamine.

**Substrate specificity**

The relative rates of oxidation of various substrates by partially purified and crude DAO preparations, (crude DAO: supernatant obtained from the homogenate by centrifugation at 10,000 x g) are shown in Table 3. Cadaverine, putrescine, hexamethylenediamine, histamine, ethylenediamine and agmatine were used as diamines, and tyramine, benzylamine, serotonin, $\beta$-phenylethylamine, n-amyamine and n-propylamine as monoamines. The rates of oxidation are expressed as percentages to that of cadaverine. With diamines, both the partially purified and the crude DAO preparation showed the highest activity with cadaverine. The activity of the partially purified DAO preparation with other substrates decreased in the following order: cadaverine, putrescine, hexamethylenediamine, histamine, ethylenediamine. Similar results were obtained with the crude DAO preparation which also catalyzed the oxidation of monoamines while the partially purified DAO preparation did not.

**Effects of inhibitors**

The effects of aminoguanidine, hydroxylamine, p-methylphenylhydrazine (MPH),

| Substrate               | Relative activity (%) | Crude DAO | Purified DAO |
|-------------------------|-----------------------|-----------|--------------|
| Cadaverine              | 100                   | 100       |              |
| Putrescine              | 82                    | 85        |              |
| Hexamethylenediamine    | 76                    | 61        |              |
| Histamine               | 38*                   | 40*       |              |
| Ethylenediamine         | 26                    | 19        |              |
| Agmatine                | 24                    | 0         |              |
| Tyramine                | 98                    | 0         |              |
| Benzylamine             | 97                    | 0         |              |
| Serotonin               | 80                    | 0         |              |
| $\beta$-Phenylethylamine| 47                    | 0         |              |
| n-Amyamine              | 45                    | 0         |              |
| n-Propylamine           | 40                    | 0         |              |
semicarbazide, KCN, iproniazid and isonicotinic acid hydrazide (INAH) on DAO activity were studied using cadaverine and histamine as substrates. The results obtained with the partially purified DAO preparation are summarized in Table 4. The pI 0 value of aminoguanidine with both cadaverine and histamine was 7.5. With hydroxylamine, MPH, semicarbazide, KCN, iproniazid and INAH as inhibitors, the pI 50 values were 6.6, 6.0, 5.8, 3.0, 2.5 and 1.6, respectively, with cadaverine and 6.5, 6.0, 5.9, 3.0, 2.4 and 1.6, respectively, with histamine. The activity-pI curves with cadaverine in the presence of the inhibitors listed above, were superimposeable on those of histamine.

**Effects of metal ions**

Table 5 shows the effects of various metal ions on the activity of partially purified DAO activity.

### Table 4. Effects of various inhibitors on partially purified DAO activity.

| Compound         | Cad.  | Hist. | Cad.  | Hist. | Cad.  | Hist. |
|------------------|-------|-------|-------|-------|-------|-------|
| Aminoguanidine   | 8.70  | 8.70  | 7.50  | 7.50  | 5.70  | 5.70  |
| Hydroxylamine    | 7.70  | 7.70  | 6.63  | 6.56  | 5.70  | 5.30  |
| P-Methylphenylhydrazine | 7.70 | 7.70 | 6.00  | 6.05  | 4.70  | 4.70  |
| Semicarbazide    | 10.00 | 9.70  | 5.83  | 5.90  | 4.70  | 4.70  |
| KCN              | 4.70  | 4.70  | 3.00  | 3.05  | 1.70  | 1.70  |
| Iproniazid       | 4.70  | 4.70  | 2.46  | 2.43  | 1.70  | 1.70  |
| INAH             | 3.70  | 3.70  | 1.63  | 1.63  | 1.00  | 1.00  |

Values are negative logarithms of the molar concentration of inhibitor. pI 0 is the minimal effective concentration, pI 50 is the concentration causing 50% inhibition and pI 100 is the concentration causing complete inactivation. The pI 50 values were obtained from activity-pI curves for the various inhibitors.

Cadaverine was used at a concentration of $3 \times 10^{-3}$ M and histamine at a concentration of $1 \times 10^{-3}$ M.

### Table 5. Effects of various metal ions on partially purified DAO activity.

| Metal       | $1 \times 10^{-5}$ M | $1 \times 10^{-4}$ M | $1 \times 10^{-3}$ M |
|-------------|----------------------|----------------------|----------------------|
|             | Cad. | Hist. | Cad. | Hist. | Cad. | Hist. |
| KNO₃        | 96   | 84    | 100  | 94    | 100  | 100   |
| NH₄Cl       | 110  | 100   | 106  | 100   | 100  | 100   |
| CuCl        | 30   | 29    | 50   | 62    | 100  | 100   |
| FeSO₄       | 97   | 100   | 100  | 100   | 100  | 100   |
| Co(NO₃)₂    | 100  | 100   | 100  | 100   | 100  | 100   |
| MgSO₄       | 100  | 100   | 100  | 100   | 100  | 100   |
| Cu(NO₃)₂    | 13   | 7     | 58   | 56    | 100  | 100   |
| Zn(NO₃)₂    | 48   | 21    | 56   | 37    | 100  | 100   |
| AlCl₃       | 0    | 0     | 89   | 100   | 97   | 100   |
| Fe(NO₃)₃    | 52   | 66    | 100  | 100   | 100  | 100   |

Values are percentages of control activity. Metal ions were used at concentrations $1 \times 10^{-5}$ M, $1 \times 10^{-4}$ M and $1 \times 10^{-3}$ M. Concentrations of cadaverine and histamine were $3 \times 10^{-3}$ M and $1 \times 10^{-3}$ M, respectively.
DAO with cadaverine and histamine as substrates. Metal ions were tested at concentrations of $1 \times 10^{-3} \text{ M}$ to $1 \times 10^{-5} \text{ M}$. At a concentration of $1 \times 10^{-4} \text{ M}$, complete inactivation was observed with $\text{AlCl}_3$, $87\%$ inhibition with $\text{Cu(NO}_3\text{)}_2$, $70\%$ inhibition with $\text{CuCl}$ and about $50\%$ inhibition with $\text{Zn(NO}_3\text{)}_2$ or $\text{Fe(NO}_3\text{)}_2$, while $\text{KNO}_3$, $\text{NH}_4\text{Cl}$, $\text{FeSO}_4$, $\text{Co(NO}_3\text{)}_2$, and $\text{MgSO}_4$ had no effects on the activity. At a concentration of $1 \times 10^{-3} \text{ M}$ $\text{CuCl}$, $\text{Cu(NO}_3\text{)}_2$, $\text{Zn(NO}_3\text{)}_2$ and $\text{AlCl}_3$ did not influence DAO activity. The inhibitory effects of these metals were similar to cadaverine and histamine.

**Effects of SH-reagents**

The effects of the SH-reagents, p-chloromercuribenzoate (PCMB), iodoacetoamide, $\text{HgCl}_2$, $\text{AgNO}_3$ and $\text{CdSO}_4$ on the activity of partially purified DAO were studied. The SH-reagents were tested at concentrations of $1 \times 10^{-8} \text{ M}$ to $1 \times 10^{-1} \text{ M}$ with cadaverine and histamine as substrates. The results are summarized in Table 6. The activity with either cadaverine or histamine decreased to approx. $10\%$ that of control value in the presence of $1 \times 10^{-3} \text{ M}$ PCMB, iodoacetoamide or $\text{CdSO}_4$. Addition of $1 \times 10^{-2} \text{ M}$ $\text{AgNO}_3$ caused about $50\%$ inhibition of activity with cadaverine and complete inhibition with histamine as substrate. Addition of $1 \times 10^{-4} \text{ M}$ $\text{AgNO}_3$ caused $15\%$ inhibition with cadaverine and $70\%$ with histamine. The effects of $\text{AgNO}_3$ on the activity of partially purified DAO are shown in Fig. 4. The $\text{pI}$ 50 value was 3.12 with cadaverine and 4.20 with histamine.

**Thermal inactivation**

Preparations of DAO in $0.1 \text{ M phosphate buffer, pH 7.1}$ in Erlenmeyer flasks were heated for 30, 60, 90 and 120 min at

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**TABLE 6. Effects of SH-reagents on partially purified DAO activity.**

| SH-reagent  | $1 \times 10^{-8} \text{ M}$ | $1 \times 10^{-4} \text{ M}$ | $1 \times 10^{-5} \text{ M}$ |
|-------------|-------------------------------|-------------------------------|-------------------------------|
|             | Cad.  | Hist. | Cad.  | Hist. | Cad.  | Hist. |
| PCMB        | 87.1  | 84.2  | 92.9  | 98.0  | 98.6  | 100   |
| Iodoacetoamide | 83.5  | 90.4  | 95.2  | 100   | 100   | 100   |
| $\text{HgCl}_2$ | 0     | 0     | 70.0  | 75.0  | 97.6  | 100   |
| $\text{AgNO}_3$ | 45.0  | 0     | 85.0  | 30.0  | 95.0  | 95.0  |
| $\text{CdSO}_4$ | 91.7  | 88.9  | 100   | 100   | 100   | 100   |

Values are percentages of control activity.

SH-reagents were used at concentrations of $1 \times 10^{-3} \text{ M}$, $1 \times 10^{-4} \text{ M}$ and $1 \times 10^{-5} \text{ M}$. Concentrations of cadaverine and histamine were $3 \times 10^{-3} \text{ M}$ and $1 \times 10^{-2} \text{ M}$, respectively.

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**Fig. 4. Effects of $\text{AgNO}_3$ on partially purified DAO activity.** Cadaverine $3 \times 10^{-3} \text{ M}$ (open circles) and histamine $1 \times 10^{-2} \text{ M}$ (solid points) were used as substrates. Abscissa, negative logarithm of molar concentration of $\text{AgNO}_3$. Ordinate, DAO activity expressed as a percentage of the control activity.
65°C or 70°C. After the heating flasks had been quickly chilled in ice water, and residual activity was determined at 38°C with cadaverine and histamine as substrates. As shown in Fig. 5 (left), marked inactivation of DAO activity was observed after 15 min incubation at 70°C. On heating for longer periods, the DAO activity remained constant and the residual activity was estimated as approx. 20% with histamine and 10% with cadaverine. In this experiment, inactivation was much stronger when measured with cadaverine than with histamine. Fig. 5 (right) shows the time course of enzyme inactivation at 65°C. Results with cadaverine and histamine were different. After incubation at 65°C for 120 min, DAO activity with histamine was approx. 40% of the original activity, while that with cadaverine was 20%.

**DISCUSSION**

There have been many reports (1-11) regarding the features of histaminase. Discrepancies among the results are mainly due to differences in the methods used for measurement of enzyme activity (14). In this study, enzyme activity was assayed by measuring oxygen consumption in the initial first min of reaction using cadaverine and histamine as substrates.

The ratio of the activities with these two substrates which had been partially purified by incubation at 60°C for 40 min differed from the ratio of a crude preparation. This suggests that heat treatment may not result in any change in the character of the enzymes themselves, but may cause a slight change in the conformation of the protein moieties.

From studies on substrate specificity Suzuki et al. (15) reported that benzylamine and n-amylamine which are substrates of MAO, were oxidized by partially purified DAO. However, results in present study were different, and may be attributed to the method used for measuring activity. Suzuki et al. measured DAO activity manometrically and
the activities with benzylamine and n-amyldamine were only 8% and 1%, respectively, as compared to cadaverine. Zeller et al. (16) also reported that DAO does oxidize various monoamines, but their enzyme preparation was crude. The present partially purified preparation did not oxidize monoamines, since MAO was inactivated by heat treatment (18).

Among the inhibitors tested aminoguanizine was most inhibitory, followed by hydroxylamine, p-methylphenylhydrazine and semicarbazide. These results are the same as those reported by Zeller (1).

The partially purified enzyme was completely inhibited by $1 \times 10^{-3} \text{M HgCl}_2$, but not affected by $1 \times 10^{-5} \text{M HgCl}_2$. The SH-group of this enzyme may not be essential for activity (17, 18). The inhibitory effect of AgNO$_3$ differed depending on whether histamine or cadaverine was used as substrate. Addition of $2 \times 10^{-4} \text{M AgNO}_3$ caused complete inhibition with histamine as substrate, but only 10% inhibition was observed with cadaverine.

Heat treatments at 65°C and 70°C also had different effects on the activities of cadaverine and histamine. The partially purified enzyme was relatively heat stable with histamine as substrate, but was inactivated markedly with cadaverine.

These results suggest that the present partially purified enzyme preparation contains two enzymes, one catalyzing oxidation of cadaverine and the other catalyzing oxidation of histamine. If so, the properties of these two enzymes are very similar although they differ in certain enzymic characters, such as susceptibilities to AgNO$_3$ and heat stability.

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