# OXIDATION OF 1,4-DIAMINO-2-BUTENE TO PYRROL, A SENSITIVE TEST OF DIAMINE OXIDASE ACTIVITY

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*Dedicated to Professor F. Šantavý on the occasion of his 60th birthday.*

Diamine oxidase from hog kidney and pea oxidizes 1,4-diamino-2-butene (dehydroputrescine) to pyrrol. One mol of $O_2$ per mol of substrate is consumed during the reaction and a stoichiometric quantity of $H_2O_2$ and $NH_3$ is formed. The effects of pH, concentration of enzyme and substrate on the reaction rate are described. The new substrate is oxidized up to 1·4-times faster by the animal enzyme and at a 7·3-times slower rate by the plant enzyme than putrescine under optimum conditions. The reaction of enzymatically formed pyrrol with *p*-dimethylaminobenzaldehyde gives rise to a colored product with absorbance maximum at 563 nm; this permits the activity of animal diamine oxidase to be measured with a high sensitivity. The method is suitable for diagnostic purposes in the analysis of human blood serum. An improved method of preparation of dehydroputrescine as well as some of its characteristics are also described.

Diamine oxidase (EC 1.4.3.6) has become an object of increasing interest in clinical diagnostics since the time the changes in its level were shown to indicate disturbances in pregnancy, renal and pulmonary diseases as well as other pathological states[1]. There are a few methods only permitting low activities of this enzyme in blood serum to be detected and even the use of these methods is connected with some problems. The so far most sensitive isotope method[2], which measures the radioactivity of the oxidation product of [$^{14}$C]-putrescine after its extraction with toluene, is limited of access and still expensive. The spectrophotometric method reported recently[3] and based on measurement at 250 nm of the product of oxidation of p-dimethylaminomethylbenzylamine, is suitable for solutions of low protein content. Lastly, in the optical test[4] determining ammonia by a coupled reaction with glutamate dehydrogenase, difficulties are encountered when crude, unpurified samples are analyzed[1].

We found in our studies on the substrate specificity of diamine oxidase that a substrate analog, 1,4-diamino-2-butene (dehydroputrescine), is readily oxidized by the enzyme and that the oxidation product can be easily determined photometrically with a high sensitivity. The reaction was studied with diamine oxidase of animal and plant origin and the results obtained with the two model systems are described in this paper.

## EXPERIMENTAL

### 1,4-Diamino-2-butene Dihydrochloride

A suspension of 259 g (1·4 mol) of potassium phthalimide in 600 ml of dimethylformamide was treated with 139 g (0·65 mol) of 1,4-dibromo-2-butene, added in parts within 15 min with stirring and cooling by water. The reaction was completed by heating the mixture 1 h at 100°C. The latter was poured into 4·5 l of water and cooled to 0°C. The precipitate of 1,4-diphthalimido-2-butene was filtered off with suction, washed with water (yield 200·6 g, 89·1%), and recrystallized from boiling glacial acetic acid. M.p. 233°C corr. (Langenbeck and coworkers[5] report an uncorrected m.p. 226–227°C).

1,4-Diphthalimido-2-butene (101 g) was refluxed 60 h with a mixture of 500 ml of glacial acetic acid and 500 ml of 37% hydrochloric acid. The mixture was concentrated *in vacuo* and phthalic acid which had crystallized was filtered off by suction. Upon the addition of ethanol to the filtrate, 1,4-diamino-2-butene dihydrochloride precipitated (yield 38·7 g, 83·5%). The latter was dissolved in water and recrystallized by the addition of ethanol. The product decomposes over 310°C without melting. For $C_4H_{12}Cl_2N_2$ (159·1) calculated: 30·20% C, 7·60% H, 17·61% N, found: 30·52% C, 7·81% H, 17·22% N. The NMR spectrum in $D_2O$ showed 2 maxima at $\delta$ 3·66 p.p.m. ($N^+—CH_2—C=$) and 7·25 p.p.m. ($—CH=$); their ratio was 2 : 1. The product was uniform when subjected to paper chromatography in the system phenol–1M-HCl and was stained gray-blue with ninhydrin. $R_F$ 0·26 (putrescine $R_F$ 0·29). The hydrogen uptake during hydrogenation using $PtO_2$ as catalyst was 0·98 mol $H_2$ per mol of dihydrochloride. The product of hydrogenation was chromatographically and as dipicrate identical with putrescine. The dibenzoyl derivative of 1,4-diamino-2-butene melted at 178–179°C, as recorded in literature[6].

### Material

Purified diamine oxidase from shoots and cotyledons of pea of specific activity 3·4 U/mg (25°C, pH 7·0, 3·3 mM 1,4-diamino-2-butene as substrate) and hog kidney diamine oxidase of specific activity 160 milliunits (38°C, pH 9, 3·3 mM 1,4-diamino-2-butene as substrate) were prepared as described earlier[7]. Crystalline bovine liver catalase was from Reanal, Budapest, bovine serum albumin from Mann Research Laboratories, U.S.A. The remaining chemicals were of analytical reagent grade.

### Reagents and Methods

*Ehrlich's reagent*: *p*-Dimethylaminobenzaldehyde (15 g) is dissolved in 50 ml of 1-propanol and 20 ml of 37% HCl. The volume of the solution is made up to 100 ml with 1-propanol. The solution is filtered through glass paper. The reagent is stable for at least one month at room temperature.

*Standard pyrrol solution*: Pyrrol (33·5 mg, 0·5 mmol, b.p. 130°C), freshly distilled, is dissolved in 25 ml of ethanol and the solution is kept at 0°C. An aliquot of the stock solution is diluted 400-times with water before use (1 ml corresponds to 50 nmol of pyrrol).

*The standard test of diamine oxidase activity* is carried out as follows. A test tube containing 2·8 ml of buffer, 50 μg of catalase, and 3·3 mM 1,4-diamino-2-butene is thermostated at the teperature required. The reaction is started by the addition of 0·2 ml of freshly diluted enzyme and stopped 5–10 min later by adding 2 ml of Ehrlich's reagent. The colour is allowed to develop exactly 30 min in a water bath at 50°C. The solution is then cooled in an ice bath; its absorbance

at 563 nm is read within 30 min against a blank solution not containing the substrate. If the absorbance exceeds 0·7—0·8, the sample is diluted by the blank solution. The units are calculated from the absorbance values as follows:

$$\text{activity (U/ml enzyme)} = \frac{A_{563} \cdot 10^3 \cdot v}{\varepsilon \cdot d \cdot t \cdot e},$$

where $v$ is the final volume of the reaction mixture, $\varepsilon = 63000\text{M}^{-1}\,\text{cm}^{-1}$, $d$ is the length of the optical path, $t$ the time of the enzymatic reaction in min, and $e$ the volume of the enzyme solution used. One unit (U) is defined as the amount of diamine oxidase catalyzing the formation of 1 μmol of pyrrol per 1 min under the following conditions: 0·1M Tris-HCl buffer of pH 9·0, $38 \pm 0{\cdot}1$°C for hog kidney diamine oxidase, 0·1M sodium phosphate buffer of pH 7·0, $25° \pm 0{\cdot}1$°C for the enzyme from pea. For routine and kinetic measurements, a set of reaction mixtures was prepared in which the reaction was started and stopped stepwise at 15 s intervals.

*Oxygen uptake* was measured by an oxygen electrode[8] in 3 ml of reaction mixture saturated with air (concentration of $O_2$ 0·24 mM at 25°C and 0·19 mM at 38°C).

*Ammonia* was determined by the conventional microdiffusion technique of Conway[9].

## RESULTS

*Stoichiometry of reaction and identification of product*: The measurement of oxygen uptake in the presence of an excess of pea diamine oxidase at pH 7 showed that 448 nmol of oxygen was consumed for the oxidation of 450 nmol of 1,4-diamino-2-butene (Fig. 1). Following the addition of catalase, 222 nmol of oxygen was released back into the solution; this shows that a stoichiometric quantity of hydrogen peroxide arises in the process of substrate oxidation. Similar results were obtained when

TABLE I

Formation of Ammonia during Enzymatic Oxidation of 1,4-Diamino-2-butene

The substrate was oxidized successively by two portions of pea diamine oxidase (a total of 2·2 U in 0·4 ml of 0·15M phosphate, pH 7, 30 min at 20°C); ammonia formed was determined by microtitration with 0·01M-$KHI_2O_6$ after alkalization and 16 h diffusion[9] into 1 ml of 1% $H_3BO_3$.

| Experiment | Substrate μmol | $NH_3$-detd.[a] μmol | Mol. ratio $NH_3$/substrate |
|---|---|---|---|
| 1 | 2·00 | 2·19 | 1·10 |
| 2 | 3·00 | 3·11 | 1·04 |
| 3 | 4·00 | 4·35 | 1·09 |

[a] Mean value obtained by 2—4 determinations.

a smaller quantity of substrate was used (Fig. 1). Likewise, the quantity of ammonia liberated, determined in separate experiments after the oxidation of 2–4 μmol of 1,4-diamino-2-butene, corresponded on the average to 1·08 μmol of $NH_3$ per each μmol of substrate (Table I). These results show that the oxidation of 1,4-diamino-2-butene by diamine oxidase follows this equation:

$$R—CH_2NH_2 + O_2 + H_2O \rightarrow RCHO + H_2O_2 + NH_3 .$$

The final oxidation product could be isolated by distillation with steam. The first portions of the distillate contained a compound whose spectrum consisted of a simple absorption band with a maximum at 206 nm. The aqueous solution of pyrrol ($\varepsilon_{206}$ = = 5900M$^{-1}$ cm$^{-1}$) shows a characteristic absorption in the same range. The cold distillate gave a red color with the Ehrlich's reagent. This colored solution showed a spectrum with a sharp maximum at 563 nm (shoulder of 535 nm), identical to the spectrum of the colored solution which yields an authentic sample of pyrrol. These findings provide evidence that pyrrol is the final product of 1,4-diamino-2-butene oxidation by diamine oxidase.

*Reaction with Ehrlich's reagent*: The appropriate concentrations of the components in the reagent were determined in preliminary experiments. Its volume (2 ml)

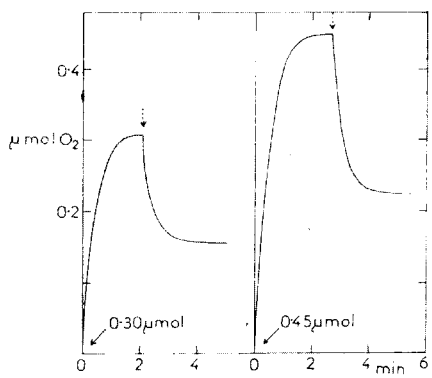



FIG. 1

Oxygen Uptake and Hydrogen Peroxide Formation during Oxidation of 1,4-Diamino-2-butene (denoted by arrow) by Excess of Pea Diamine Oxidase (2·2 U, 0·1M phosphate buffer pH 7, 25°C)

Catalase (25 μg) was added as marked by a dashed arrow.

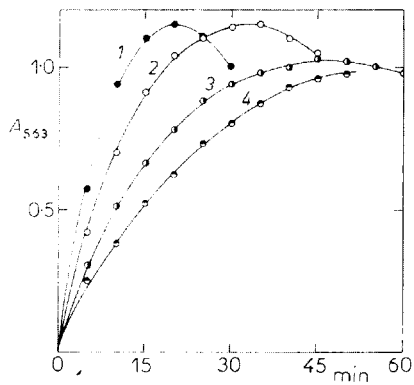


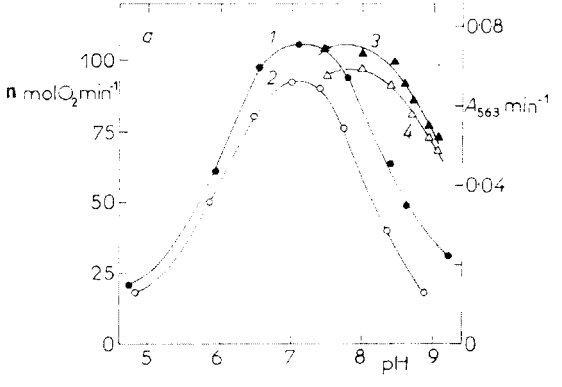

FIG. 2

Time Profile of Color Development at Different Temperatures

1,4-Diamino-2-butene (10 mM) was oxidized 5 min in 3 ml of 0·1M phosphate, pH 7, at 38°C by pea diamine oxidase (7 mU); the development of the color was examined after the addition of the Ehrlich's reagent (2 ml) at 55°C *1*, 50°C *2*, 45°C *3*, and 40°C *4*.

was chosen so that *p*-dimethylaminobenzaldehyde might not be precipitated after the addition of the aqueous pyrrol solution (3 ml). When the reagent described here was used, maximum color intensity of the pyrrol solution was obtained after 15–20 min at 20°C. The reaction mixture containing enzymatically oxidized 1,4-diamino-2-butene, *i.e.* without distillation gave the same color, yet very slowly. To terminate the reaction within reasonable time, the time dependence of color development at different temperatures was studied. As shown in Fig. 2, the time necessary for the development of maximum absorbance decreases with increasing temperature, the stability of the color, however, simultaneously decreases. The temperature of 50°C at which the reaction is completed within 30 min, was chosen for routine analyses. The color is sufficiently stable after cooling. The absorbance decreases linearly by 5·6% per hour at 20–23°C. In an ice bath the stability of the color is even better.

In an effort to determine the molar absorbance coefficient, 0·3 μmol of 1,4-diamino-2-butene was oxidized by an excess (1·1 U) of pea diamine oxidase with simul-



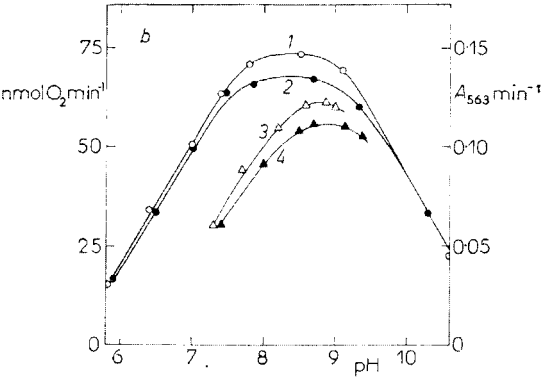




FIG. 3

Effect of pH on Oxidation of 1,4-Diamino-2-butene by Diamine Oxidases

The enzymatic activity was determined spectrophotometrically (full points) and by oxygen uptake measurement (open points) in 0·1M sodium phosphate buffers (*1*,*2*) and 0·1M Tris buffers (*3*,*4*) in the presence of 50 μg of catalase. Reaction conditions: *a* enzyme from pea, 25°C, 10 mM substrate, *b* enzyme from hog kidney, 38°C, 5 mM substrate.

taneous oxygen uptake control (Fig. 1). An aliquot of 0·1 – 0·5 ml of the reaction mixture was subjected to standard spectrophotometric analysis. The plot of absorbance measured *versus* quantity of substrate oxidized shows that the color complies with Bouguer–Lambert–Beer's low up to an absorbance value of 0·7. The molar absorbance coefficient $\varepsilon_{563} = 6{\cdot}0 \,.\, 10^4 \text{M}^{-1}\,\text{cm}^{-1}$ was read from the slope; this value is in good agreement with that obtained with a standard pyrrol solution ($\varepsilon_{563} = 6{\cdot}30 \,.\, 10^4 \text{M}^{-1}\,\text{cm}^{-1}$) and determined by the same method.

*Kinetics of oxidation*: The effect of pH on the rate of oxidation of 1,4-diamino--2-butene by pea and hog kidney diamine oxidase was examined spectrophotometrically in 0·1M sodium phosphate and Tris-buffer respectively. The enzyme activity was determined in duplicates and the pH-values were measured in separate reaction mixtures in the absence of the Ehrlich's reagent. For comparison, the effect of pH was investigated also in terms of oxygen uptake in the presence of a considerably larger quantity of the enzyme. As obvious from Fig. 3, the profile of pH-curves obtained by the two methods is practically identical. The pH-profile of the activity of amine oxidases in phosphate buffer has a typical bell shape, yet the pH-optima of both enzymes considerably differ. The pH-optimum of the enzyme from pea lies at pH 7·1 (25°C) and the animal enzyme shows a pH-optimum of 8·4 (38°C). The pH-optima

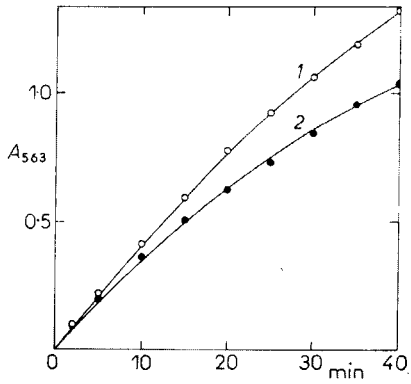



FIG. 4

Time Profile of Oxidation of 1,4-Diamino--2-butene

The enzymatic activity of pea diamine oxidase *1* was measured in 0·1M sodium phosphate buffer, pH 7, at 25°C, and of hog kidney diamine oxidase 2 in 0·1M Tris-HCl buffer, pH 9, at 38°C. At the time intervals shown 2 ml of Ehrlich's reagent were added and the color was developed 30 min at 50°C.

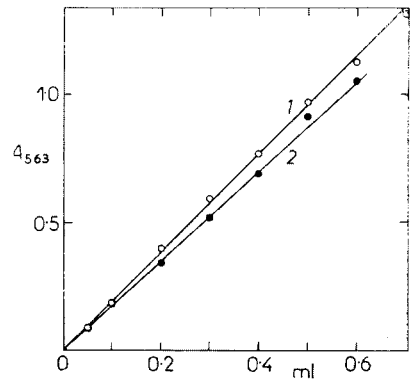



FIG. 5

Diamine Oxidase Activity as Function of Enzyme Concentration

1,4-Diamino-2-butene (3,3 mM) was oxidized by pea diamine oxidase, 5 min at 25°C and pH 7 (*1*; 30 mU/ml) and by animal diamine oxidase 5 min at 38°C and pH 9 (2; 28 mU/ml).

are shifted approximately by half-a-unit of pH toward the alkaline range in Tris buffer.

The dependence of absorbance on time of incubation with 3·3 mM 1,4-diamino-2-butene shows a linear profile at optimum pH for the first 10 min at least (Fig. 4). The time curve levels off with increasing time only slowly and thus even very low enzymatic activities can be measured spectrophotometrically with relative reliable results when incubating enzyme with the substrate for long periods. Linearity was achieved up to activities of 17 and 21 mU with both enzymes in experiments in which the dependence of absorbance on enzyme amount was measured after 5 min incubation (Fig. 5).

The data obtained by measurement of initial oxidation rate $v$ at a variable substrate concentration $s$ showed that the interaction of diamine oxidase with 1,4-diamino-2-butene follows the Michaelis kinetics up to approximately 3 mM concentration. This substrate concentration is sufficient for the saturation of the enzyme. The values of apparent Michaelis constants were read from a $1/v$ *versus* $1/s$ plot: $K'_m = 1{\cdot}9 \cdot 10^{-5}$M (0·1M Tris, pH 9, 38°C) for hog kidney diamine oxidase, $K'_m = 5{\cdot}4 \cdot 10^{-5}$M (0·1M phosphate, pH 7, 25°C) for pea diamine oxidase. Both amine oxidases are inhibited by high concentrations of 1,4-diamino-2-butene (Fig. 6) like by other diamines[10]. In view of the theory of enzyme-bisubstrate complex formation, the inhibition was expressed according to Dixon and Webb[11] by plotting $1/v$ *versus* $s$. When the linear dependence was extrapolated for $1/v = 0$, the following values of apparent substrate-inhibitor constants were obtained: $K'_s = 0{\cdot}121$M for the animal enzyme, $K'_s = 0{\cdot}146$M for the plant enzyme. The values of all constants are means of 2–3 independent measurements.

The rate of oxidation of 1,4-diamino-2-butene and putrescine was compared in terms of oxygen uptake measured in sodium phosphate and Tris buffers. The data given in Table II show that the animal enzyme oxidizes 1,4-diamino-2-butene

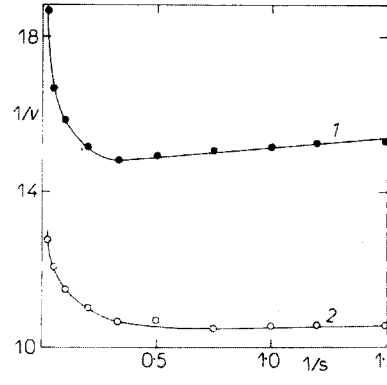



FIG. 6

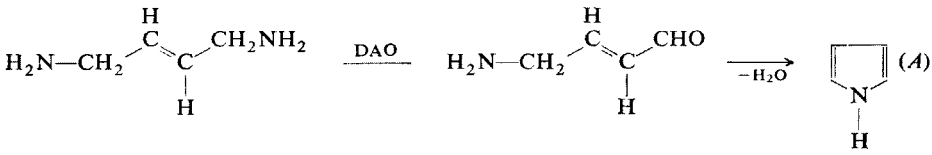
Inhibition of Diamine Oxidase by Excess of 1,4-Diamino-2-butene

The reciprocal initial rate $1/v$ is expressed as a change of $A_{563}^{-1}$ min and plotted *versus* reciprocal concentration of 1,4-diamino-2-butene $1/s$ (in $mM^{-1}$). The activity of pea diamine oxidase was measured at 25°C and pH 7 (*1*), of hog kidney diamine oxidase at 38°C and pH 9 (*2*).

approximately 1·2–1·4-times faster than putrescine under conditions close to optimum conditions. 1,4-Diamino-2-butene is however a worse substrate as regards the enzyme from pea. The rate of oxygen uptake was approximately 7·3–8·8-times lower than with putrescine. The activity calculated from the oxygen uptake is in good agreement with the activity measured spectrophotometrically (Table II).

## DISCUSSION

We have shown in this study that 1,4-diamino-2-butene undergoes oxidative deamination by diamine oxidase (DAO) to pyrrol. The latter is most likely formed *via* an intermediary product, *i.e. via* cyclization of 4-amino-2-buten-1-al. We explain the strikingly slow development of the color during the reaction of the Ehrlich's reagent with the oxidation product of 1,4-diamino-2-butene compared to pyrrol by assuming that the amino aldehyde might have been predominantly of *trans* configuration which hinders the cyclization (equation (*A*)).




The sterical arrangement of 1,4-diamino-2-butene can indirectly be deduced from the fact that the compound has been prepared from 1,3-butadiene by addition of bromine and then by a procedure not leading to a sterical change on the double bond; some data on 1,4-dichloro-2-butene, also prepared from butadiene, indicate *trans* configuration[15]. The slow cyclization of the amino aldehyde is also most likely the cause of another phenomenon: if an excess of diamine oxidase is allowed to act on a limitting quantity of 1,4-diamino-2-butene, the substrate is oxidized quantitatively yet the enzyme looses its activity simultaneously. Since the reaction mixture can inactivate another portion of enzyme added and pyrrol itself is without effect on the enzyme, it is obvious that the inhibition is caused by the primary oxidation product. There is no inhibition at low enzyme concentrations and in the presence of an excess of substrate. A small quantity of amino aldehyde formed in this case is most likely displaced from the active center of the enzyme by the substrate.

The method developed in this study for the spectrophotometric determination of diamine oxidase activity makes use of an extremely sensitive reaction between pyrrol, the end product, and *p*-dimethylaminobenzaldehyde. We found in preliminary experiments that of the various, modified Ehrlich reagents the most suitable for our purposes was that containing perchloric acid and 1-propanol. The reaction was allowed to proceed 15 min at 60°C (ref.[12]). Under these conditions, however, oxidized 1,4-diamino-2-butene yielded a colored product only partly, the pH-optimu-

ma did not agree with the values determined from oxygen uptake and were shifted up to half-a-unit of pH toward the alkaline range. Our modified reagent containing hydrochloric acid gives a more intensive color. When purified diamine oxidase was used and even when bovine serum albumin (up to 24 mg) was added, entirely clear solutions were obtained and albumin did not affect the color intensity. After the addition of 0·1 – 0·3 ml of human blood serum, turbidity was observed; the latter could be removed only after 1 ml of 10% trichloroacetic acid had been added after the development of the color and cooling. If necessary, the volume of all components of the reaction mixture can be proportionally reduced. Continuous shaking in order to saturate the reaction mixture with oxygen[13] is without effect on the rate of the enzymatic reaction.

Judging by the value of the molar absorbance coefficient only, our method for animal diamine oxidase is 10-times more sensitive than the optical test of Lorenz

TABLE II

Oxidizability of 1,4-Diamino-2-butene and Putrescine

The rate of oxygen uptake was measured in 3·0 ml of 0·1M buffer in the presence of 50 μg of catalase by an oxygen electrode and calculated per 1 ml of dilute enzyme used. (An uptake of 1 nmol of $O_2$/min corresponds to enzyme activity of 2 mU). The approximate optimum conditions of oxidation of the substrates are set in italics.

| Substrate | Substrate concentration mM | Final pH | nmol $O_2$/min | Relative rate % |
|---|---|---|---|---|
| | Pea diamine oxidase (25°C) | | | |
| Putrescine | 10·0 | 7·0[a] | 270·0 | 100·0 |
| 1,4-Diamino-2-butene | 10·0 | 7·0[a] | 30·7 | 11·4 |
| Putrescine | 10·0 | 7·0[a] | 260·0 | 100·0 |
| 1,4-Diamino-2-butene | 10·0 | 8·0[b] | 32·3 | 12·4 |
| Putrescine | 3·3 | *7·0*[a] | 107·4 | 100·0 |
| 1,4-Diamino-2-butene | 3·3 | *7·0*[a] | 14·6[c] | 13·6 |
| | Hog kidney diamine oxidase (38°C) | | | |
| Putrescine | 3·3 | *7·4*[a] | 16·7 | 100·0 |
| 1,4-Diamino-2-butene | 5·0 | *8·5*[a] | 24·3 | 145·5 |
| Putrescine | 3·3 | *7·4*[a] | 16·7 | 100·0 |
| 1,4-Diamino-2-butene | 5·0 | *8·9*[b] | 20·3 | 121·5 |
| Putrescine | 3·3 | 8·9[b] | 6·1 | 100·0 |
| 1,4-Diamino-2-butene | 3·3 | 8·9[b] | 13·3[d] | 218·0 |

[a] Sodium phosphate buffer; [b] Tris-HCl buffer; [c] 30·3 mU determined spectrophotometrically; [d] 27·8 mU determined spectrophotometrically

and coworkers[4] and approximately 6-times more sensitive than the method of Bardsley and coworkers[3], measuring the oxidation of *p*-dimethylaminomethylbenzylamine to the corresponding aldehyde ($\varepsilon_{250} = 11000\text{M}^{-1}\,\text{cm}^{-1}$). The new method of determination of enzymatic activity is approximately 3-times more sensitive than the *o*-aminobenzaldehyde method[13,14] even for pea diamine oxidase which oxidizes 1,4-diamino-2-butene much slower than putrescine. One international unit corresponds to the quantity of enzyme which in 1 min in a final volume of 5 ml will give rise to a color of $A_{563} = 12{\cdot}6$ ($d = 1$ cm); under the conditions described here, 1 unit of hog kidney diamine oxidase corresponds to 0·8 putrescine unit (pH 7·4, 38°C), 1 unit of pea diamine oxidase to 7·3 putrescine units (pH 7·0, 25°C). The reproducibility of the determination is very good: a variation coefficient of $\pm 0{\cdot}97\%$ at an absorbance value $0{\cdot}614 \pm 0{\cdot}006$ was obtained in 10 concurrent experiments in which the purified enzyme from pea was pipetted manually.

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