17-ketones. The failure of urinary chloride to induce these reactions was attributed to the inhibitory effect of native urea.

This work was done during the tenure of an Empire Rheumatism Council Fellowship by one of us (J.K.N.).

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Factors which Interfere in the Manometric Assay of Monoamine Oxidase

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The reaction which monoamine oxidase is believed to catalyse may be represented by

$$R.CH_2.NH_2 + H_2O + O_2 \rightarrow R.CHO + H_2O_2 + NH_3,$$

in which one molecule of oxygen is absorbed for each molecule of substrate oxidized. When tyramine is used as substrate the theoretical oxygen uptake is apparently obtained, the observed value in the present experiments being 1·9 atoms/molecule of tyramine. Previous authors (Kohn, 1937; Pugh & Quastel, 1937; Blaschko, Richter & Schlossman, 1937; Luschinsky & Singher, 1945) have pointed out that crude monoamine oxidase preparations also contain catalase, cytochrome *c* and cytochrome oxidase, aldehyde oxidase and peroxidase, enzymes whose combined action (see Fig. 1) would be

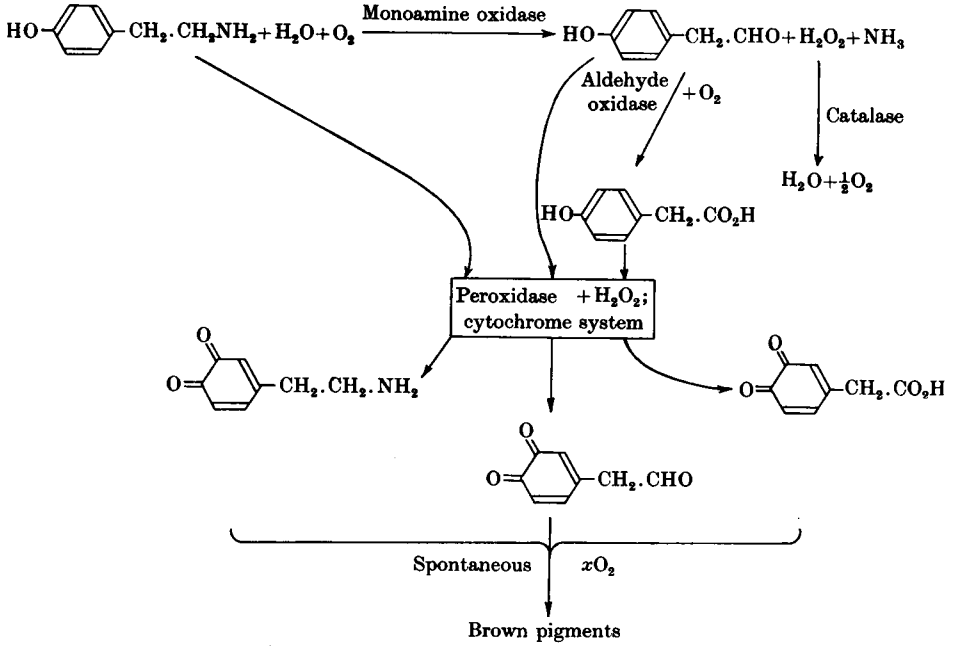

Fig. 1. Reactions likely to occur when tyramine is incubated with crude monoamine oxidase.

expected to make the observed oxygen uptake different from the theoretical value, and seriously to interfere with monoamine oxidase determinations based upon oxygen-uptake measurements.

It was shown by Blaschko *et al.* (1937) and Kohn (1937) that the side oxidations could be inhibited and the oxygen uptake with tyramine as substrate decreased to one atom/molecule by the addition of 10^{-2} M semicarbazide and 10^{-3} M cyanide. This value is only one-half of that to be expected if peroxidase, catalase, aldehyde oxidase and cytochrome oxidase were inhibited.

As the manometric method was needed for routine assays of the monoamine oxidase activity of mitochondrial preparations it was considered advisable to investigate the reasons for this discrepancy before placing any reliance on the results. Experiments were therefore performed to discover (*a*) whether the interfering enzymes indicated in Fig. 1 were in fact present in mitochondrial suspensions and (*b*) how their activity would be affected by various inhibitors.

EXPERIMENTAL

Mitochondrial suspensions. Mitochondria were separated from rat-liver homogenates by the method of Hawkins (1952), washed twice with distilled water and suspended in distilled water so that 2 ml. of suspension was equivalent to 1 g. of fresh liver. This preparation was free from blood pigments and soluble protein.

Oxygen uptake. Uptake of O_2 during deamination was measured manometrically at 38°, readings being taken at 0, 10, 20 and 30 min. for an activity determination and, when total O_2 uptake was being measured, at 30 min. intervals thereafter until O_2 ceased to be absorbed. The composition of the basic reaction mixture was as follows. Main compartment: enzyme preparation (1 ml.), 0·24 M sodium phosphate buffer (pH 7·0; 0·2 ml.), water as necessary; side arm: 0·1 M amine (0·2 ml.); centre well: 2 M-KOH (0·1 ml.), filter-paper strip; if KCN was present in the main compartment 2 M-KOH was replaced by 2 M-KCN, which was sufficiently alkaline to absorb CO_2. The final volume of the reaction mixture was adjusted to 2 ml. with water. Additions to this mixture were made by replacing some of the water in the main compartment, all solutions except 2 M-KOH and 2 M-KCN being adjusted to pH 7·0 before use. The flasks were gassed with O_2 for 2 min. and equilibrated for 10 min. before tipping. A control reaction mixture containing no substrate was also prepared and any O_2 absorbed by it was subtracted from that absorbed in the test reaction mixtures.

Catalase. This was measured by the following modification of the method of Goldacre & Galston (1953). The reaction mixture contained: 0·24 M sodium phosphate buffer, pH 7·0 (1 ml.), mitochondrial suspension (2·5 ml.), 0·1 M-H_2O_2 (1 ml.) and water to 10 ml. The reaction was carried out in boiling tubes incubated at 38° and was started by adding the H_2O_2. Samples (2 ml.) were removed at 15, 30, 45 and 60 min. and transferred to tubes containing 2 ml. of 5% trichloroacetic acid + 0·5 ml. of 12 N-H_2SO_4. Precipitated protein was removed by filtration, the precipitate washed with water and the H_2O_2 in the combined filtrate and washings titrated with 0·1 M-$KMnO_4$. The residual H_2O_2 was expressed as a percentage of that originally present and plotted against the time of sampling. The effect of cyanide on catalase activity was studied by replacing some of the water in the reaction mixture by cyanide solution adjusted to pH 7·0.

Peroxidatic activity of catalase. This was demonstrated by adding 0·2 ml. of 4% ethanol to the main compartment of the Warburg flask in the basic reaction mixture.

Cytochrome oxidase. This was measured by the method of Schneider & Potter (1943), in which O_2 uptake is followed during the oxidation of ascorbic acid by a mixture of cytochrome oxidase and cytochrome *c*. The effects of inhibitors were investigated by including appropriate solutions in the main compartment of the Warburg flask.

Aldehyde oxidase. This was measured by the method of Walkenstein & Weinhouse (1953), in which O_2 uptake is followed when mitochondria are incubated at 37° and pH 7·4 with 0·01 M aldehyde, 0·1 M-KCl, 0·005 M-$MgSO_4$, 10^{-4} M fumarate, 10^{-3} M adenosine triphosphate (ATP) and 0·02 M sodium phosphate buffer.

Chemicals. These were British Drug Houses, Ltd. laboratory grade except Antabuse (tetraethylthiuram disulphide) from L. Light and Co. Ltd., ATP (Boots Ltd.), and Marsilid (1-*iso*nicotinyl-2-*iso*propylhydrazine) from Roche Products Ltd.

RESULTS

Cytochrome oxidase

An active cytochrome oxidase was found to be present in the washed mitochondrial suspension. The curves in Fig. 2 show that in the conditions used the activity was dependent on the concentration of added cytochrome *c*, in the absence of which cytochrome oxidase activity could not be observed. This agrees with the results of Friedenwald & Herrmann (1942) who also found that tissue preparations could readily be obtained which contained abundant cytochrome oxidase but relatively little cytochrome *c*.

Cyanide (10^{-3} M) was found to inhibit the enzyme completely (Fig. 2); however, it it unlikely that this would explain the effect of cyanide in lowering the O_2 uptake in the basic reaction mixture since the cytochrome oxidase of washed mitochondria is almost inactive in the absence of added cytochrome *c*.

Semicarbazide (0·01 M) did not affect mitochondrial cytochrome oxidase and its ability to lower the O_2 uptake during the deamination of tyramine must therefore be due to some other effect (Fig. 2).

When 10^{-4} M Marsilid, a potent monoamine oxidase inhibitor (Zeller & Barsky, 1952) which does not inhibit cytochrome oxidase, was added to the basic reaction mixture the O_2 uptake was reduced to zero. This indicates that, even in the absence of cyanide, oxidation of tyramine by the cytochrome system (see Fig. 1) does not occur.

The evidence is therefore against the cytochrome system being operative in washed mitochondria.

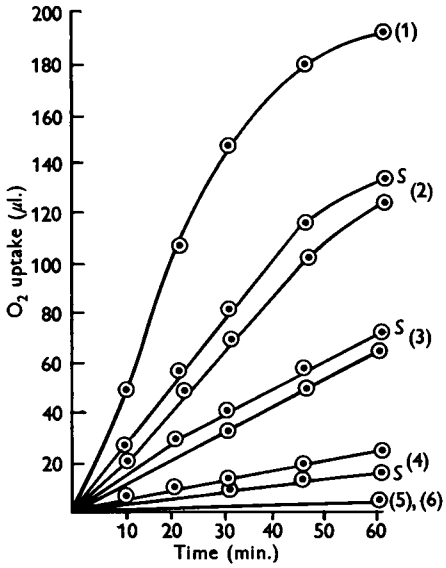

Fig. 2. Effect of 0·01 M semicarbazide on the cytochrome oxidase activity of washed mitochondria at different concentrations of cytochrome *c*. Mitochondrial suspension (1·0 ml.) was incubated at 37° with 0·2 ml. of 0·24 M sodium phosphate, pH 7·4, 0·2 ml. of 0·004 M-$AlCl_3$, 0·2 ml. of 0·114 M ascorbic acid, 0·1 ml. of 2 M-KOH on filter-paper strip, and the following additions: (1) cytochrome *c*, 1 mg./ml.; (2) cytochrome *c*, 0·3 mg./ml.; (3) cytochrome *c*, 0·1 mg./ml.; (4) cytochrome *c*, 0·01 mg./ml.; (5) cytochrome *c*, 1 mg./ml. $+10^{-3}$ M-KCN; (6) no cytochrome *c*. Curves marked '*S*' show the effect of adding 0·01 M semicarbazide. Final vol., 2 ml. Gas phase, O_2.

Aldehyde oxidase

The presence of aldehyde oxidase in washed mitochondria was suggested by the fact that the addition of semicarbazide to the basic reaction mixture, with tyramine as substrate, was accompanied by a marked fall in O_2 uptake. Blaschko *et al.* (1937) suggested that this was due to binding of the aldehyde formed during deamination and its consequent protection against attack by aldehyde oxidase.

Soluble aldehyde oxidase has been described by Gordon, Green & Subrahmanyan (1940), and by Racker (1949), and a diphosphopyridine nucleotide-dependent aldehyde oxidase associated with mitochondria has been described by Walkenstein & Weinhouse (1953). The soluble aldehyde oxidase would not be expected to remain associated with mitochondria washed with distilled water, and in view of the disruptive effects of distilled water on mitochondria it is possible that Walkenstein & Weinhouse's insoluble enzyme might be inactivated by removal of the diphosphopyridine nucleotide upon which its activity depends. All attempts to demonstrate aldehyde oxidase in washed mitochondria were without success, as the following experiments will show.

The absence of aldehyde oxidase in washed mitochondria was suggested by the fact that Antabuse (4 µg./ml.), which inhibits liver aldehyde oxidase (Kjeldgaard, 1949), was found to be without effect on the O_2 uptake when included in the basic reaction mixture.

Aldehyde oxidase activity in the mitochondrial suspension was then measured by the method of Walkenstein & Weinhouse (1953). Redistilled acetaldehyde, crotonaldehyde or *iso*valeraldehyde was used as substrate, and in no case was O_2 found to be absorbed over a period of 3 hr.

The failure to demonstrate the presence of aldehyde oxidase may have been due to (*a*) the presence of inhibitors in the aldehydes (Walkenstein & Weinhouse, 1953), or (*b*) the inactivation of the enzyme by a high initial concentration of aldehyde (Kjeldgaard, 1949). In the following experiment both of these factors were eliminated by supplying acetaldehyde continuously at low concentration by coupling the oxidation of ethanol with the production of H_2O_2 in the monoamine–oxidase reaction (see below).

Four reaction mixtures were set up, two without and two with semicarbazide. Ethanol was added to one of each pair and the O_2 uptake followed in each reaction mixture.

In the absence of semicarbazide the extra O_2 uptake resulting from the addition of ethanol includes O_2 used in the oxidation of ethanol to acetaldehyde by peroxidase, plus any that may be used in the subsequent oxidation of the acetaldehyde to acetic acid by aldehyde oxidase.

Table 1. *Effect of semicarbazide on the extra oxygen uptake resulting from coupling the oxidation of ethanol with the deamination of tyramine by monoamine oxidase*

Each vessel contained: side arm—0·1 ml. of 0·1 M tyramine; centre well—0·1 ml. of 2 M-KOH and a filter-paper slip; main compartment—1 ml. of mitochondrial suspension, 0·2 ml. of 0·24 M sodium phosphate buffer (pH 7·0) and, where present, 0·2 ml. of 4% (v/v) ethanol and 0·2 ml. of M or 0·1 M semicarbazide; total vol., 2 ml.; O_2 gas phase, temp. 38°.

Expt.	Semicarbazide (M)	Time (min.)	Extra O_2 uptake due to addition of ethanol (µl.): Semicarbazide absent	Extra O_2 uptake due to addition of ethanol (µl.): Semicarbazide present
1	0·01	100	87	106
		200	100	111
		300	113	120
2	0·10	100	86	94
		200	94	93

In the presence of semicarbazide, which traps the acetaldehyde and prevents its conversion into acetic acid, the extra O_2 absorbed after the addition of ethanol is used only in the oxidation of ethanol to acetaldehyde. Therefore, if aldehyde oxidase is present the extra O_2 uptake in the absence of semicarbazide should be greater than that in the presence of semicarbazide by a factor of approximately two. Alternatively, if aldehyde oxidase is absent, the extra O_2 absorbed after the addition of ethanol should be independent of the presence of semicarbazide. The experiment was performed with 0·1 M and 0·01 M semicarbazide; within the limits of error neither concentration was found to influence the extra O_2 absorbed after the addition of ethanol (Table 1). It was therefore concluded that aldehyde oxidase was absent.

Catalase and peroxidase

By the method of Goldacre & Galston (1953) a high concentration of catalase was found to be present in the washed mitochondria. Even in the presence of 0·05 M cyanide the catalase activity was not inhibited completely (Fig. 3). Semicarbazide (0·01 M) was found to be without effect on the catalase activity. Goldacre & Galston (1953) showed that 2×10^{-6} M 2:4-dichlorophenol would inhibit crystalline catalase by 50%; however, the results shown in Fig. 3 indicate that mitochondrial catalase activity is not affected by this inhibitor.

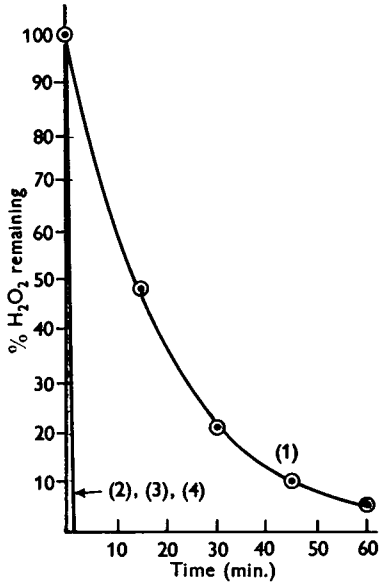

Fig. 3. Effect of cyanide or 2:4-dichlorophenol on the catalase activity of washed mitochondria. 0·01 M-H_2O_2 was incubated at 37° and pH 7·0 with mitochondrial suspension and (1) 0·05 M-KCN; (2) 10^{-3} M-KCN; (3) $0{\cdot}5 \times 10^{-4}$ M 2:4-dichlorophenol; (4) no inhibitor.

Table 2. *Effect of cyanide, azide or semicarbazide on the coupled oxidation of ethanol*

Each Warburg vessel contained: side arm—0·1 ml. of 0·1 M tyramine; centre well—0·1 ml. of 2 M-KCN or 2 M-KOH, filter-paper slip; main compartment—0·1 ml. of 0·24 M sodium phosphate buffer, pH 7·0, and the additions shown; total vol., 2 ml. O_2 gas phase, temp. 38°.

Additions (final concn.)	O_2 uptake* after 6 hr. (μl.)
None	210
Ethanol (0·4%, v/v)	324
Ethanol (0·4%, v/v) + KCN (0·001 M)	116
KCN (0·001 M)	116
Ethanol (0·4%, v/v) + NaN_3 (0·01 M)	210
NaN_3 (0·01 M)	210
Ethanol (0·4%, v/v) + semicarbazide (0·01 M)	217
Semicarbazide (0·01 M)	122

* Calc. for 1 atom of O_2/mole of tyramine = 112 μl.

The mitochondrial suspensions were found to be capable of coupling the formation of H_2O_2 during the deamination of tyramine with the oxidation of ethanol. This is in agreement with the experiments of Keilin & Hartree (1945), who showed that enzymically liberated H_2O_2 could be used in coupled oxidations.

This peroxidatic effect was demonstrated by adding 0·4% ethanol to the basic reaction mixture, when an increase in the O_2 uptake equivalent to one atom/molecule of substrate occurred. Similar experiments were performed by Kohn (1937), who used a crude monoamine oxidase preparation from pig liver. This increase in O_2 uptake on adding ethanol indicates that hardly any of the H_2O_2 liberated in the basic reaction mixture is used in coupled oxidations involving tyramine or its primary oxidation product. It must therefore be concluded that because of the absence of a suitable substrate the peroxidase effect does not operate during deamination of tyramine by mitochondria. The coupled oxidation was inhibited by 10^{-3} M cyanide or by 0·01 M azide but not by 0·01 M semicarbazide (Table 2).

Effect of various experimental conditions on O_2 uptake during monoamine oxidase activity

The effects of ethanol, azide, cyanide and semicarbazide on the total O_2 uptake during the deamination of tyramine or *iso*pentylamine were investigated. The results, together with the reactions which are thought to occur under each set of conditions, are illustrated in Table 3.

In some cases the observed O_2 uptake was greater than the theoretical based on the activity of enzymes shown to be present in the washed mitochondria. The extra O_2 is represented by x and was found to be equivalent to 0·8–0·9 atom/molecule of substrate.

Table 3. *Total O_2 uptake during the deamination of tyramine and isopentylamine*

	Atoms of O_2/molecule of substrate					
	Tyramine (10^{-2}M)			*iso*Pentylamine (10^{-2}M)		
Combination*	Theoretical†	Observed	Reaction† involved	Theoretical	Observed	Reaction† involved
M	$1+x$	1·9	D.Ca.Sp	1	0·7	D.Ca
M.C	1	1·1	D.Ca	1	0·9	D.Ca
M.Sm	1	1·1	D.Ca	1	0·95	D.Ca
M.Sm.C	1	1·0	D.Ca	1	0·9	D.Ca
M.A	$1+x$	1·9	D.Ca.Sp	1	0·9	D.Ca
M.Et	$2+x$	2·9	D.P.Sp	2	2·3	D.P‡
M.Et.C	1	1·1	D.Ca	1	1·1	D.Ca‡
M.Et.Sm.C	1	1·0	D.Ca	1	1·1	D.Ca‡
M.Et.A	$1+x$	1·8	D.Sp	1	—	—
M.Et.Sm	2	1·9	D.P	2	2·1	D.P‡

* M, mitochondria; C, 10^{-3}M-KCN; Sm, 10^{-2}M semicarbazide; A, 10^{-2}M sodium azide; Et, 0·4% (v/v) ethanol.
† D, deamination; Ca, catalase; P, peroxidase; Sp, spontaneous oxidation; x, O_2 uptake due to spontaneous oxidation of *p*-hydroxyphenylacetaldehyde.
‡ In these cases O_2 uptake was not complete after 5 hr. and the O_2 absorbed at that time is therefore expressed as a fraction of the O_2 absorbed in a reaction mixture containing only mitochondria and buffered *iso*pentylamine (M).

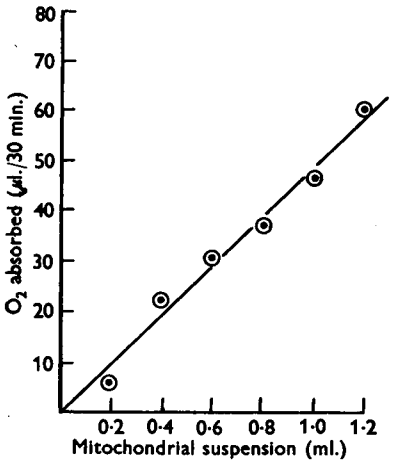

Fig. 4. Relation between enzyme concentration and O_2 uptake in the recommended monoamine oxidase activity test.

Method for routine monoamine oxidase determination

On the basis of the preceding experiments, monoamine oxidase activity was measured by following O_2 uptake during the deamination of tyramine in the presence of 10^{-2}M semicarbazide and 10^{-3}M cyanide. Under these conditions only monoamine oxidase and catalase appear to be active, spontaneous oxidations are inhibited, and O_2 is absorbed at the rate of one atom/molecule of tyramine deaminated. Tyramine is rapidly oxidized in comparison with other readily obtainable amines, O_2 uptake is linear over the first 30 min. of the reaction and is also directly proportional to the monoamine oxidase concentration (Fig. 4). The composition of the recommended reaction mixture was as follows: main compartment—enzyme preparation (1 ml.), 0·24M sodium phosphate buffer, pH 7·0 (0·2 ml.), 0·01M-KCN (0·2 ml.), 0·1M semicarbazide (0·2 ml.), water (0·2 ml.); side arm—0·1M tyramine (0·2 ml.); centre well—2M-KCN (0·1 ml.), filter-paper strip. All solutions except the 2M-KCN were adjusted to pH 7·0.

DISCUSSION

Of the enzymes included in Fig. 1 as being able to modify the uptake of oxygen during the deamination of tyramine by monoamine oxidase, cytochrome oxidase, peroxidase and catalase have been shown to be present in washed rat-liver mitochondria. Cytochrome oxidase was inactive because of the absence of sufficient cytochrome *c*, and peroxidase appeared to be inactive in the monoamine oxidase activity tests because of the absence of a suitable substrate. Aldehyde oxidase could not be detected in the washed mitochondria. Catalase was particularly active in all the mitochondrial preparations, and in a reaction mixture containing only buffered tyramine and washed mitochondria would be expected to reduce the oxygen uptake to one atom/molecule of substrate oxidized:

$$R.CH_2.NH_2 + H_2O + O_2 \xrightarrow{\text{monoamine oxidase}} R.CHO + H_2O_2 + NH_3$$

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + \tfrac{1}{2}O_2.$$

This was found not to be the case, however, the observed oxygen uptake being 1·9 atoms/molecule of substrate oxidized.

The addition of 10^{-3}M cyanide or 10^{-2}M semicarbazide to the basic reaction mixture decreased

the oxygen uptake to the theoretical value of one atom/molecule of tyramine oxidized. At the concentrations used these two substances are known not to inhibit either monoamine oxidase (Blaschko *et al.* 1937) or catalase. Other enzymes likely to be affected by 10^{-3}M cyanide were found to be absent from washed mitochondria or inactive under the conditions used for deamination. Semicarbazide (10^{-2}M) was found to be without effect on any of the enzymes against which it was tested. It would, therefore, appear that these substances may inhibit a spontaneous oxidation, possibly the spontaneous oxidation of *p*-hydroxyphenylacetaldehyde, the primary deamination product of tyramine. This compound is known to be unstable in air (Langheld, 1909) and both cyanide and semicarbazide could prevent its spontaneous oxidation; semicarbazide by the formation of a semicarbazone, and cyanide by heavy-metal binding or by cyanohydrin formation. The oxidation of this compound would account for the extra oxygen uptake observed in Table 3.

*iso*Valeraldehyde, the primary deamination product of *iso*pentylamine, was found not to be susceptible to further oxidation under the conditions of the monoamine oxidase assay; consequently, cyanide or semicarbazide would not be expected to lower the oxygen uptake during *iso*pentylamine deamination, and this was found to be the case (see Table 3). Attempts to prepare *p*-hydroxyphenylacetaldehyde were unsuccessful and its properties and behaviour under the conditions of the monoamine oxidase assay could, therefore, not be investigated.

SUMMARY

1. The occurrence in washed rat-liver mitochondria of enzymes likely to modify the oxygen uptake during the oxidative deamination of *iso*pentylamine or tyramine has been studied.

2. Catalase and peroxidase have been shown to be present and active. Peroxidase was inhibited by 0·01M azide and by 10^{-3}M cyanide. Catalase was not inhibited by 2×10^{-6}M 2:4-dichlorophenol or by 0·01M semicarbazide; it was incompletely inhibited by 0·05M cyanide.

3. Cytochrome oxidase was present but inactive owing to the absence of sufficient cytochrome *c*. The enzyme was inhibited by 10^{-3}M cyanide.

4. Aldehyde oxidase could not be detected.

5. The effects of cyanide, semicarbazide, azide and ethanol on oxygen uptake during the deamination of tyramine and *iso*pentylamine have been studied, and the results discussed.

6. It is concluded that a buffered reaction mixture containing the monoamine oxidase preparation, 0·01M tyramine, 0·01M semicarbazide and 10^{-3}M cyanide would be suitable for measuring monoamine oxidase activity. Under these conditions one atom of oxygen was absorbed for each molecule of substrate oxidized and the oxygen absorbed at 30 min. was proportional to the monoamine oxidase concentration.

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