THE DETERMINATION OF MONOAMINE OXIDASE ACTIVITY

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DEFINITION OF AMINE OXIDASES

According to Zeller[1,2], the oxidative deamination of amines consists in the substitution of carbonyl groups for aliphatic amino groups. The enzymes which catalyse this oxidative deamination are usually called amine oxidases or dehydrogenases.

The amine oxidases are divided in two main groups: the first group consisting of those which attack α-methylamines (*e.g.* amphetamine), and the second group of those which do not do so.

Two categories are distinguishable in the second group: those enzymes which are inhibited in the presence of 10^{-3} M semicarbazide, and those which are not. The monoamine oxidases belong to this last group of " 10^{-3} M semicarbazide resistant oxidases ". The different groups of amine oxidases are listed in *Table 1*.

Table 1. Amine oxidases

Oxidation of α-methylamines	*No oxidation of α-methylamines*
Amphetamine oxidase	Monoamine oxidase (MAO) Diamine oxidase (DAO) Benzylamine oxidase Mescalin oxidase Spermine oxidase
Inhibition by 10^{-3} M *semicarbazide*	*No inhibition by* 10^{-3} M *semicarbazide*
Diamine oxidase Spermine oxidase Benzylamine oxidase Mescalin oxidase	Monoamine oxidase

ACTION OF AMINE OXIDASES

Primary and secondary as well as tertiary amines (*e.g.* hordenine) are attacked[1]. Most enzymes belonging to this group have a pH maximum near 7·4. At this pH only small amounts of the substrate are found in the form of free base; the substrate can, therefore, be represented as being in ionic form[3]. The over-all reaction may be represented by the following equation:

$$RCH_2N^+HR'R'' + O_2 \longrightarrow RCH{:}N^+R'R'' + H_2O_2$$
$$RCH{:}N^+R'R'' + H_2O \longrightarrow RCHO + N^+H_2R'R''$$
$$H_2O_2 \xrightarrow{\text{Catalase}} H_2O + \tfrac{1}{2}O_2$$

$$RCH_2N^+HR'R'' + \tfrac{1}{2}O_2 \longrightarrow RCHO + N^+H_2R'R''$$

(R, R′ and R″ = H or alkyl)

Evidence for the disappearance and occurrence of the reaction products given above abounds in the literature (see reviews by Zeller[1], Blaschko[4], and Davison[5]). The formation of imino derivatives is hypothetical. Through a rapid non-enzymatic hydrolysis, such a product would produce the aldehyde and a lower amine. As early as 1937, Richter[3,6] pointed out this possibility by analogy with the action of amino-acid oxidase.

DETERMINATION OF AMINE OXIDASE ACTIVITY

The various substances involved in the reaction are discussed consecutively below, together with the possibilities which they afford for the determination of the enzymatic activity.

Disappearance of substrate, $RCH_2N^+HR'R''$

When the enzymatic activity is measured by means of the disappearance of the substrate, it is desirable to use as low a substrate concentration as possible at the start of the reaction to ensure that there is sufficient reduction of the concentration for reliable measurement. On the other hand, a large excess of the substrate is generally preferred in enzyme determinations because, if low concentrations are used, the reaction products formed may inhibit the enzymatic activity. If the enzyme activity is to be measured by following the decrease of substrate concentration over a period of time, it is recommended that, as a check, the formation of a reaction product during the same period should also be determined. An example of this is the simultaneous determination of the rates of the disappearance of tryptamine and of the formation of ammonia, described below (see p. 487).

Five different methods are used for the determination of the substrate concentration, and these are discussed briefly below.

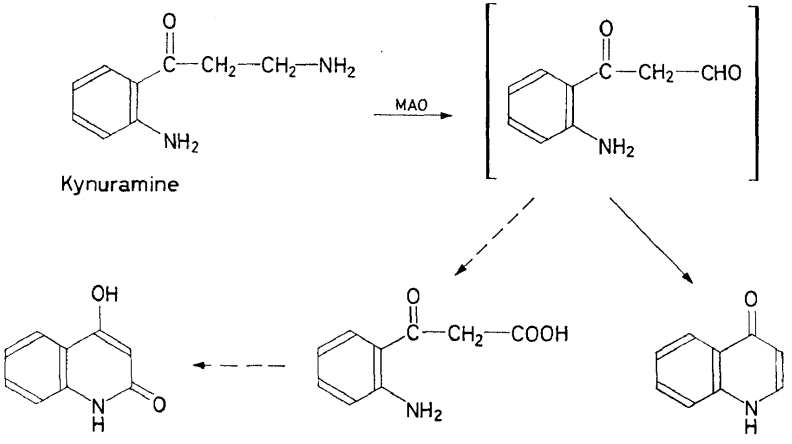
Biological method—Many physiologically active substances, such as adrenalin, serotonin, and tryptamine, are satisfactory MAO substrates. Very sensitive physiological determinations have been described for these substances[7]: effects on blood pressure, on contractions of the rat uterus (0·0001 μg adrenalin), on blood vessels of the rabbit ear (0·000002 μg), on intestinal muscle, *etc.* Although these methods can be very specific, they are difficult to carry out and require considerable technical skill.

Pharmacological method—The potentiation of the convulsant action of tryptamine *in vivo* by a variety of pharmacological agents facilitates the measurement of MAO activity in terms of its inhibitory activity[8-10] on this effect (as a result of the destruction of tryptamine).

Colorimetric method—Good substrates are aliphatic amines (not the lower members of the series) and amines with an aromatic ring[1]. The organic

bases can be extracted from tissues and determined colorimetrically by a methyl orange reaction[11]. The use of tyramine[12-14] or serotonin[15-17] as substrate permits the most specific determinations.

Spectrophotometric method—A very interesting method has been described by Weissbach[18]. Kynuramine, a specific substrate of MAO, shows a characteristic ultra-violet light absorption spectrum with a maximum at 366 mμ. The enzymatic deamination produces an aldehyde which, through intramolecular (non-enzymatic) condensation, leads to the formation of a quinoline derivative which shows no absorption at 366 mμ (*Scheme 1*). Both of the reaction products shown in *Scheme 1* could be formed; however, intramolecular non-enzymatic condensation of the amino-aldehyde proved to be faster than the further oxidation of the aldehyde to the acid or lactam[18].



Scheme 1. Metabolic changes during incubation of kynuramine with rat liver homogenate (Weissbach[18])

The reduction of absorption as a function of time is taken as the measure of enzymatic activity.

According to our experiments, this method appears to be the most rapid and simple for the measurement of MAO activity, and it is very well suited to routine determinations.

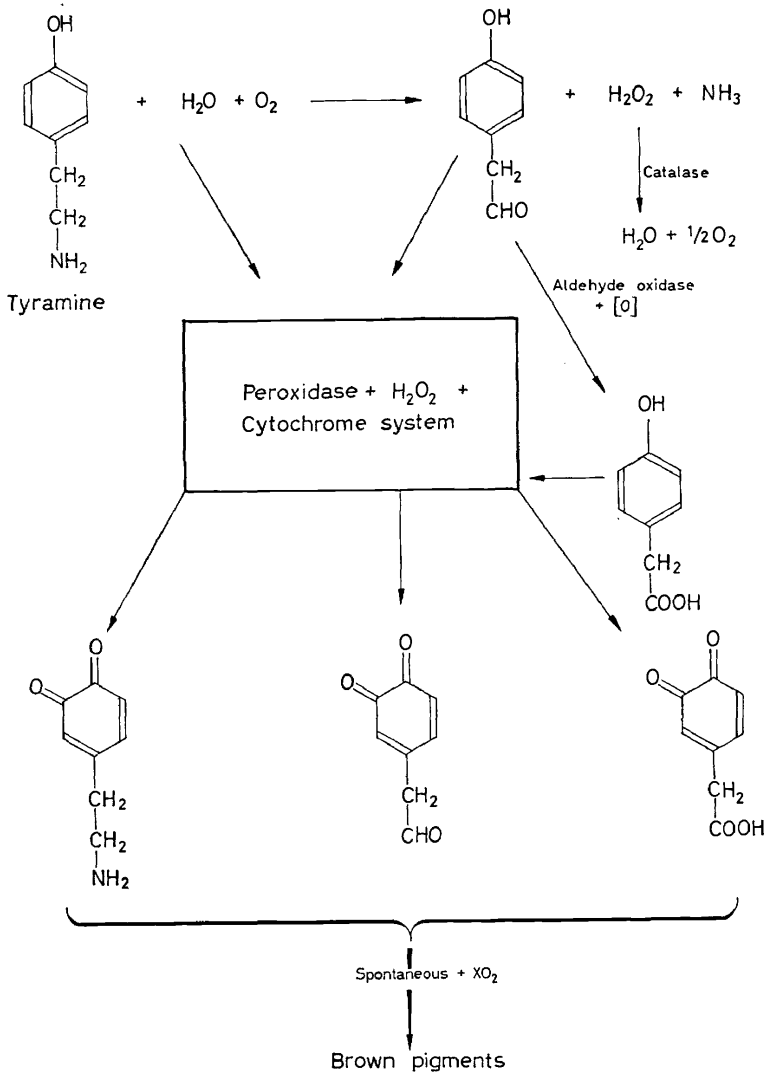
Weissbach[19] has recently described another, similar method for the determination of amino-acid oxidase.

Fluorimetric method—As a result of the development of a new apparatus[20] for fluorescence measurements, new specific determinations of biologically-active amines have been described[21]. The determinations are very sensitive: some hundredths of a microgram can be accurately determined. Methods have been described for serotonin[22,23], tryptamine[24], adrenalin and noradrenalin[23,25,26], histamine (diamine oxidase activity)[27] and others. The fluorimetric determination should be adaptable for the determination of MAO activity at the microlevel, *e.g.* on 10 mg of brain tissue.

Oxygen uptake

According to the experiments conducted by Cotzias[28-33], it appears that

only under well-defined conditions is the manometrically-measured oxygen uptake in the ratio of one atom of oxygen per molecule of substrate. A value greater than one is usually found[33] for the ratio O/NH_3.



Scheme 2. Enzymatic and non-enzymatic reactions which may occur during incubation of tyramine with rat liver homogenate (Creasey[34])

Many enzymatic or non-enzymatic side-reactions may influence this ratio, as has been pointed out by Creasey[34] (see *Scheme 2*).

The addition of 10^{-2} M semicarbazide and 10^{-3} M cyanide to the reaction mixture may give a stoichiometric ratio[24]. The sensitivity of the manometric determination is, however, very low compared with that of other

methods. A minimum amount of 25 μmol of substrate is generally used, whereas 0·5 μmol is sufficient for the ammonia determination.

Hydrogen peroxide

Zeller takes the oxidation of indigo disulphonate by the peroxide formed as the standard for enzymatic activity[35]. The decoloration of indigo disulphonate is, however, also catalysed by diamine oxidase, amino-acid oxidase, and choline oxidase; the method is not very specific, therefore.

The substitution of tetrazolium salts for oxygen as hydrogen acceptor produces intensely-coloured formazans[36]. However, when no oxygen is present, reduction is almost as rapid in the absence as in the presence of substrate, and, therefore, the reduction cannot be used for quantitative determinations.

Formation of aldehyde, RCHO

The aldehyde formed is normally oxidized further to the corresponding carboxylic acid, or converted into a resinous polymeric substance. These transformations may be prevented by adding to the reaction mixture aldehyde reagents such as cyanide or semicarbazide.

Richter[3] identifies the aldehydes as 2,4-dinitrophenylhydrazones after the addition of semicarbazide to the reaction mixture. Green and Haughton[37,38] have developed a colorimetric determination: after the conversion of the aldehyde semicarbazone to the 2,4-dinitrophenylhydrazone derivative, the colour of the compound is measured in an alkaline solution. A large excess of the substrate may be added. The reaction is very sensitive, and accurate measurement of the conversion of 0·1 μmol of substrate is possible. This method appears to be suitable for kinetic investigations.

Ammonia or a lower alkylamine

The majority of investigators use the Conway unit for the determination of ammonia and lower alkylamines[3,29], or a microapparatus for the distillation of ammonia[39]. The apparatus of Zeller[40] and Cotzias[32], with a modified Warburg reaction vessel allowing simultaneous determination of oxygen uptake and ammonia evolution, is worth mentioning.

Little or no attention has, however, been given to Seligson's apparatus[41], which was designed for the determination of ammonia in blood and has been used by Sabina[42] for the determination of amino-acid oxidase. It is equally well suited to the determination of MAO. The apparatus consists of a diffusion flask of 50 ml capacity with a glass rod fixed on the stopper (*Figure 1*). The extremity is dipped into an acid solution (sulphuric acid, citric

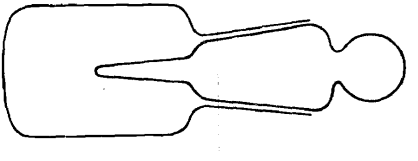


Figure 1. Diffusion flask (Seligson's apparatus)

acid, boric acid, *etc.*). After the reaction mixture has been made alkaline, the ammonia is separated by diffusion and absorbed on the glass rod. To accelerate this procedure, the diffusion flasks are placed for 45 min on a rotating wheel. After the glass rod has been washed, the determination of ammonia can be made by any colorimetric method[41-53].

METHODS

Determination using kynuramine as substrate

Tissue homogenates are prepared by homogenizing the tissue with 9 volumes of 0·5 M phosphate buffer, pH 7·4. A stock solution of substrate is obtained by dissolving 4·4 mg kynuramine dihydrobromide (Regis Chemical Co., Chicago) in 20 ml water. The disappearance of substrate as a result

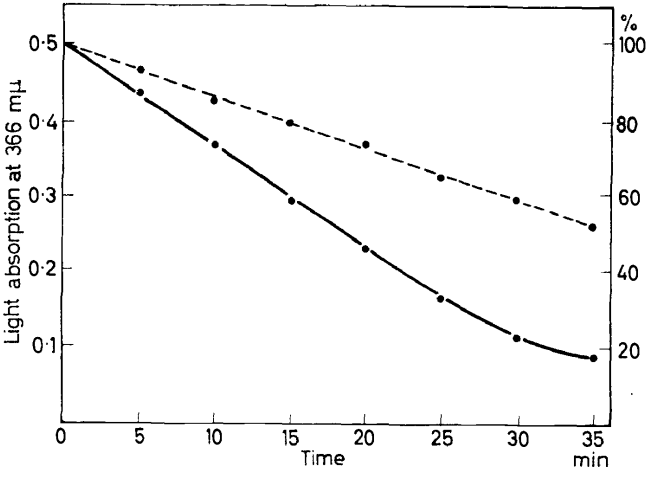


Figure 2. Decrease in light absorption at 366 mμ as a function of time during incubation of kynuramine with rat liver homogenate. The results obtained with dialysed tissue are indicated by the broken line, those with non-dialysed tissue by the full line

of MAO activity is followed by using an Eppendorf colorimeter with mercury lamp, 366 mμ filter and cuvettes of 1 cm light path.

0·3 ml 0·5 M phosphate buffer, pH 7·4, 0·4 ml homogenate (≡ 40 mg fresh tissue), water to 2·5 ml, and finally 0·5 ml kynuramine solution are mixed in a cuvette, and the fall of the light absorption at 366 mμ is measured every 5 min. A blank experiment in which water is substituted for kynuramine is always carried out.

Figure 2 shows the results of an experiment on rat liver homogenate (Wistar strain). The full line indicates the destruction with non-dialysed tissue, the broken line that with dialysed tissue. Non-dialysed tissue has much greater activity, the cause of which has not been further investigated. Weissbach[18] found an enzymatic activity in rat liver of 0·078 μmol/h per mg of protein. We have found a value of 0·060 μmol/h per mg of protein.

This method appears to be very suitable for routine determinations. The investigation of compounds which have an inhibiting or activating influence

on enzymatic activity can be followed very rapidly. Thus iproniazide[54-56], a substance which produces considerable MAO inhibition *in vitro* as well as *in vivo*, showed a 100 per cent inhibition of the activity in this experiment at a concentration of 10^{-3} M.

Determination using tryptamine as substrate: disappearance of substrate and formation of ammonia

Dialysed tissue homogenate is prepared by homogenizing the tissue with 9 volumes of 0·5 M phosphate buffer, pH 7·4, and dialysing in the cold for about 16 h against three changes of the phosphate buffer. The substrate solution used contains 2 μmol/ml tryptamine hydrochloride (Hoffman–La Roche).

0·5 ml homogenate (≡ 50 mg fresh tissue) and 0·5 ml tryptamine solution (≡ 1 μmol) are mixed in the diffusion flask, similar to that shown in *Figure 1*. After a given incubation period at 37°, the flask is opened and 2 ml saturated potassium carbonate are added, after which the flask is immediately closed. The diffusion flask is placed on a wheel which then is run for 45 min at 30 r.p.m. The ammonia absorbed on the glass rod is washed into a 10 ml tube with 2 ml 2·3 M acetate buffer solution, pH 4·41, and 2 ml " ninhydrin reagent "*. The tube is placed for 20 min in a boiling water-bath, and then cooled for 5 min in running water. Finally, the coloured solution is diluted to 10 ml with 50 per cent (v/v) aqueous ethanol, and the extinction at 578 mμ measured in a 1 cm cuvette, using an Eppendorf colorimeter with mercury lamp and 578 mμ filter.

After the diffusion of the ammonia formed, the alkaline tissue homogenate is washed with 10 ml benzene (pure, analytical grade) in a 25 ml separating funnel; 1 ml 10 N sodium hydroxide is added, and the excess tryptamine is extracted with the benzene. 1 ml of the benzene layer is added to 2·5 ml *p*-dimethylaminobenzaldehyde solution (stock solution prepared by dissolving 45g of the solid in 10 N HCl and diluting to 100 ml with the same solvent) in a 1 cm cuvette (with stopper), and the base extracted for 1 min; 10 min later the extinction is measured at 578 mμ[57]. *Table 2* lists the results

Table 2

Incubation time (min)	*Ammonia formed* (μmol)	*Tryptamine disappeared* (μmol)
10	0·17	0·20
20	0·31	0·30
30	0·43	0·45

obtained using rat liver homogenate. These results were obtained using previously established, strictly linear, calibration curves, showing that 1

* The " ninhydrin reagent " was freshly prepared each day by mixing: (a) 15 ml ninhydrin solution (stock solution made by dissolving 4g ninhydrin (Sigma) in 150 ml methylcellosolve (Eastman Kodak)); (b) 15 ml " diluted KCN solution ", obtained by diluting 2 ml " stock KCN solution " to 100 ml with methylcellosolve (" stock KCN solution " made by dissolving 65·11 mg potassium cyanide in the minimum amount of water and diluting this solution to 100 ml with methylcellosolve); (c) 50 ml 2·3M acetate buffer solution, pH 4·41 (reagents of Verbeke[58]).

μmol ammonia gives an extinction of 1·38 and that 1 μmol tryptamine gives an extinction of 0·60, at 578 mμ. The enzymatic activity using tryptamine as substrate amounts to 0·08 μmol/h per mg protein.

DISCUSSION

Various ammonia determinations described in the literature[41-53] have been carried out. The determinations using the phenol–hypochlorite reagent are very sensitive, but the reagent itself is very unstable, however. The measurements with the phenosafranin–hypobromite reagent are not sufficiently reproducible. Among the ninhydrin reactions investigated, the reagent used by Verbeke[58] was found to be the most stable and was, therefore, preferred to the others.

Tryptamine is very stable in an alkaline solution. The base may be extracted with 1 N sodium hydroxide in benzene, and further re-extracted with 1 N hydrochloric acid; the ultra-violet light absorption spectrum remains unchanged. The method is very rapid and simple. In the future, attempts will be made to determine serotonin, tyramine and histamine in a similar way, using different colour reagents. This would bring about a great simplification of the method described by Udenfriend *et al.*[12–14] for the colorimetric determination of the above-mentioned amines in biological material.

The methods described above can be adapted without difficulty for the determination of diamine oxidase activity. Using histamine as substrate, the excess of substrate can be determined in various ways (as has been reported in this paper); the ammonia liberated, as in the MAO determinations, may be used as the measure of the enzymatic activity.

In conclusion, the methods for the determination of monoamine oxidase activity have been greatly simplified by the development of the technique described above, in which the disappearance of the substrate, and the determination of the ammonia formed, may be carried out very rapidly and simply.

I wish to thank Mr Verbeke for allowing me to publish his method of ammonia determination.

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