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A Colorimetric Method for the Estimation of Monoamine Oxidase

BY A. L. GREEN* AND THELMA M. HAUGHTON
Chemical Defence Experimental Establishment, Porton Down, Salisbury, Wilts

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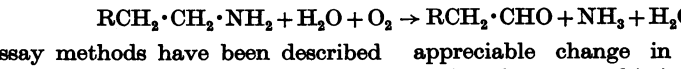
The reaction catalysed by monoamine oxidase is generally represented (Zeller, 1951; Blaschko, 1952) by the equation



$$RCH_2 \cdot CH_2 \cdot NH_2 + H_2O + O_2 \rightarrow RCH_2 \cdot CHO + NH_3 + H_2O_2. \quad (1)$$

Quantitative assay methods have been described based on measurement of the disappearance of substrate, the uptake of oxygen, or the liberation of ammonia, but none of these is entirely satisfactory for studies of the kinetics of the reaction. The most widely used method, measurement of the oxygen consumption, is relatively insensitive and is complicated by secondary reactions which occur, owing to the presence of other oxidizing enzymes, in normal tissue or mitochondrial-monoamine-oxidase preparations. The hydrogen peroxide is decomposed by catalase, which is found in all the tissue preparations, thus reducing the oxygen uptake from 2 atoms/mole of substrate as indicated in equation (1) to 1 atom/mole. The aldehyde may be further oxidized to the corresponding carboxylic acid or to resinous polymeric products; this results in a time-dependent and uncontrolled absorption of further oxygen (Cotzias & Greenough, 1959). This decomposition of the aldehyde may be prevented if aldehyde trapping agents, such as cyanide or semicarbazide, are added to the assay mixture, and under these conditions the oxygen absorption can be limited to 1 atom/mole of substrate (Creasey, 1956) but only at the expense of a further loss in sensitivity. Further, the presence of cyanide ions in the assay mixture may produce a dramatic change in the susceptibility of the enzyme to certain inhibitors (Davison, 1957). Measurement of the ammonia liberated provides a reliable and sensitive assay method but the procedure is rather laborious (Cotzias & Dole, 1951). Measurement of substrate disappearance means there must be an appreciable change in substrate concentration during the assay; this is undesirable for studies of kinetics.

The aldehyde semicarbazone, which is formed quantitatively when the enzymic oxidation is carried out in the presence of an excess of semicarbazide, can be readily converted into the corresponding 2:4-dinitrophenylhydrazone (Richter, 1937). Dinitrophenylhydrazones have an intense colour in alkaline solution (Friedemann & Haugen, 1943), and this offers a convenient means of measuring the enzymic activity. The present paper describes an assay procedure based on this dinitrophenylhydrazone formation. The method is sensitive and can be used over a wide range of substrate concentrations without significant depletion of the substrate during the reaction.

EXPERIMENTAL AND RESULTS

Materials. Guinea-pig-liver mitochondria, prepared by differential centrifuging of guinea-pig liver homogenized in 0·25M-sucrose (Hawkins, 1952), were used as the enzyme source. The sedimented mitochondria were suspended in 0·1M-phosphate buffer, pH 7·4 (83 ml. of 0·2M-Na_2HPO_4 + 17 ml. of 0·2M-NaH_2PO_4 + 100 ml. of water); 1 ml. of suspension was equivalent to 1 g. wet wt. of liver. This preparation was stored at −10°, and showed little loss of activity even after storage for several months.

Tyramine hydrochloride was obtained from L. Light and Co. Ltd., Colnbrook, Bucks.

* Present address: Smith Kline and French Research Institute, Mundells, Welwyn Garden City, Herts.

p-Hydroxyphenylacetaldehyde 2:4-dinitrophenylhydrazone, required as a colorimetric standard, was prepared as described by Richter (1937) by addition of 2:4-dinitrophenylhydrazine to the product obtained from the enzymic oxidation of tyramine in the presence of semicarbazide. On crystallization from ethanol the compound had m.p. 180° (decomp.) (Richter gives m.p. 183–186° from benzene); light-absorption max. (in ethanol) 358 mμ (ϵ 23 000). When dissolved in alkali this dinitrophenylhydrazone is at first deep red, but the colour gradually changes to orange–yellow when the solution is kept at room temperature for about an hour. This colour change is accelerated by heat and is complete if the solution is kept at 80° for 10 min. In 0·1 N-NaOH the orange–yellow form is stable at room temperature for several hours, and has a light-absorption maximum at 425 mμ (ϵ 9500).

Spectra. The u.v. spectra of the dinitrophenylhydrazone in alcohol and alkali and all other extinction measurements were determined with a Unicam SP. 500 spectrophotometer.

Assay procedure. 0·05 M-Semicarbazide hydrochloride, neutralized to pH 7·4 (1·0 ml.), 0·2 M-phosphate buffer (1·6 ml.) and enzyme (1·0 ml. of an approximately tenfold dilution of the stock suspension with buffer) were placed in a loosely stoppered 25 ml. conical flask which was shaken in a water bath at 25°. After 10 min., to allow the flask contents to reach a uniform temperature, 0·1 M-tyramine hydrochloride, neutralized to pH 7·4 (0·4 ml.), was added and the mixture was shaken in the bath for a further 30 min. 0·5 N-Acetic acid (1·0 ml.) was then added to stop the reaction. The flask contents were transferred to a centrifuge tube and heated in boiling water for 3 min. to precipitate the protein which was then removed by centrifuging. 2:4-Dinitrophenylhydrazine in 2 N-HCl (2·0 ml., 0·5 mg./ml.) was added to 2·0 ml. of the supernatant, and the mixture was kept at room temperature for 10 min. The dinitrophenylhydrazone which was formed was extracted with benzene (5·0 ml.), and 4·0 ml. of the benzene layer was then shaken with 0·1 N-NaOH (4·0 ml.). In both extraction steps the mixtures were centrifuged to ensure complete separation of the two layers. The benzene layer was then discarded, and the alkaline layer was heated at 80° for 10 min. to convert the initial red form of the dinitrophenylhydrazone into the orange–yellow form. When cool the solution was transferred to a glass 1 cm. spectrophotometer cell, and its extinction was measured at 450 mμ. After correction for the blank (a parallel run containing water instead of substrate), the extinction gave a direct measure of the enzymic activity.

Batches of six to eight estimations can be conveniently carried out at one time.

The typical results in Fig. 1 show that the extinction increases linearly both with time of incubation and with enzyme concentration. This linear relationship holds up to an extinction of at least 1, and over a time of at least 1 hr.

The reproducibility of the above procedure is illustrated by the following extinction readings (after correction for a blank of 0·084) obtained in five replicate runs: 0·636, 0·618, 0·616, 0·616, 0·606.

Extraction procedures. The double-extraction technique (cf. Friedemann & Haugen, 1943) was adopted, to eliminate as much unchanged 2:4-dinitrophenylhydrazine as possible from the final alkaline solution and so minimize the blank due to the strong colour of alkaline solutions of dinitrophenylhydrazine itself.

The completeness of the extraction of *p*-hydroxyphenylacetaldehyde 2:4-dinitrophenylhydrazone from benzene by alkali is dependent on the pH. Benzene solutions of the crystalline compound were shaken with buffers or aqueous sodium hydroxide over the pH range 11–14, and the concentration of the dinitrophenylhydrazone in both the benzene and alkaline layers was determined by measurement of the extinctions at 360 and 425 mμ respectively. A similar experiment was carried out on 2:4-dinitrophenylhydrazine. With N-sodium hydroxide, almost 100% extraction of the dinitrophenylhydrazone was achieved, but the stronger alkali also produced a marked rise in the blank reading.

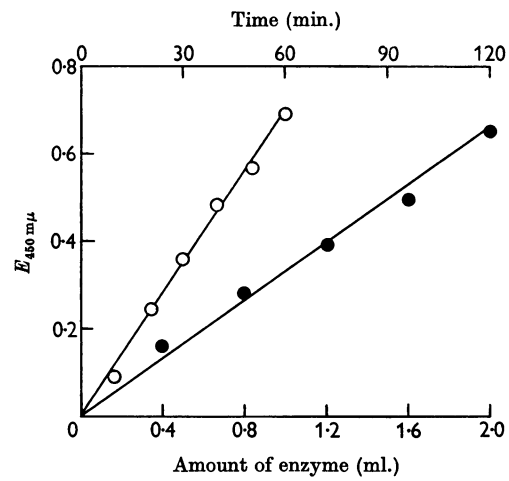


Fig. 1. Dependence of extinction on time and enzyme concentration. ○: Enzyme suspension (0·6 ml. of an eightfold dilution of the stock suspension), tyramine (0·01 M) and semicarbazide (0·0125 M) in a total volume of 4 ml., incubated for various times at pH 7·4 and 25°; ●: enzyme suspension (a 20-fold dilution of the stock suspension), tyramine (0·01 M) and semicarbazide (0·0125 M) in a total volume of 4 ml., incubated for 30 min. at 25° and pH 7·4.

0·1 N-Sodium hydroxide which extracted 91–93 % of the dinitrophenylhydrazone from the benzene gave the most satisfactory compromise between completeness of extraction and minimization of the blank.

The efficiency of the extraction of the dinitrophenylhydrazone into benzene during the assay was investigated as follows. Two flasks were run in parallel as in the assay method, one a test sample with substrate, the other a blank without substrate. After deproteinization, samples from each of the two flasks were mixed in known proportions. 2:4-Dinitrophenylhydrazine was then added and the dinitrophenylhydrazone was extracted as usual, first into benzene and then into alkali. A second extraction of the mixtures with benzene and alkali produced very little further colour, and the ratios of the extinctions of the alkaline extracts from the first and second benzene extractions, after correction for the blanks, showed that the first extraction was about 90 % complete. The corrected extinctions of the first alkaline extracts were directly proportional to the fraction of test sample, and hence to the concentration of dinitrophenylhydrazone in each mixture.

These results, taken in conjunction with the reproducibility of the assay procedure itself and the linear dependence of extinction on enzyme concentration (Fig. 1), show that although the overall extraction process is only about 80 instead of 100 % complete, the extinctions of the final alkaline solutions give a direct measure of the dinitrophenylhydrazone which has been formed.

Stoicheiometry. In one experiment the assay was carried out in Warburg flasks fitted with manometers instead of in the usual conical flasks. The reaction was stopped after 45 min. when 7·2 μg.-atoms of oxygen had been absorbed. The remainder of the assay procedure was then carried out in the normal way. The amount of dinitrophenylhydrazone recovered was 5·8 μmoles. After correction for the incompleteness of the extraction, this represents a total dinitrophenylhydrazone production of about 7·3 μmoles, i.e. 1 μmole of dinitrophenylhydrazone for each μg. atom of oxygen absorbed.

Other factors influencing the colour. Variation in the concentration of dinitrophenylhydrazine used in the assay procedure showed that an increase above the chosen value of 0·5 mg./ml. did not enhance the intensity of the colour formation but merely increased the blank. Any appreciable decrease below 0·5 mg./ml. did, however, cause some diminution in the extinction of the final solution.

The concentration of semicarbazide that was chosen (0·0125 M) was the minimum consistent with efficient trapping of the aldehyde. A large increase in semicarbazide concentration (> 0·05 M) caused a slight decrease in the extinction.

The activity of the enzyme was not significantly affected by variation in the concentration of the tyramine substrate provided this was not decreased below 1 mM. When the reciprocal of the extinction was plotted against the reciprocal of the substrate concentration over the range 1–0·1 mM, a straight line was obtained consistent with the enzymic oxidation obeying the Michaelis–Menten rate equation ($v = k[\mathrm{E}]\,[S]/(K_m + [S])$) with a value of 3×10^{-4} moles/l. for K_m.

DISCUSSION

Some of the difficulties which arise when current methods for monoamine-oxidase estimation are used in studies of kinetics were described in the introduction. Measurement of the rate of aldehyde formation, by first trapping the aldehyde with semicarbazide and then converting the semicarbazone into the corresponding 2:4-dinitrophenylhydrazone, enables many of these difficulties to be avoided. The technique is reasonably quick and straightforward. The method is reproducible and gives satisfactory linear relationships between extinction and enzyme concentration or time, as illustrated in Fig. 1.

The double-extraction procedure is desirable to minimize the amount of unchanged dinitrophenylhydrazine present in the final alkaline solution, the extinction of which is measured on the spectrophotometer. The extraction process does not entirely eliminate dinitrophenylhydrazine, but the small amount left can be adequately corrected for by reference to the blank run. Since the absorption peak of the dinitrophenylhydrazine itself in alkali is at about 390 mμ it was found to be preferable for assay purposes to measure the extinction of the dinitrophenylhydrazone at 450 mμ instead of at the peak wavelength of 425 mμ. This results in only a small decrease in the extinction of the dinitrophenylhydrazone, but to a considerable fall in the blank reading.

When allowance is made for the incompleteness of the two extraction steps, there is a one to one correspondence between the number of μg.atoms of oxygen absorbed and the number of μmoles of dinitrophenylhydrazone formed. If the assay procedure is carried out as described, a final extinction of 0·1 (after correction for a blank of the same magnitude) represents an oxygen uptake of about 2 μl. and the destruction of less than $\frac{1}{200}$ of the tyramine substrate.

SUMMARY

1. A method is described for the estimation of monoamine oxidase based on measurement of the aldehyde formed during the enzymic oxidation of tyramine. The unstable aldehyde is prevented from decomposing by the presence of semicarbazide in

the assay mixture. The semicarbazone so formed is then converted into the corresponding 2:4-dinitrophenylhydrazone. The colour of this material in alkaline solution provides a sensitive measure of the enzymic activity.

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The Biochemistry of Aromatic Amines

8. SYNTHESIS AND DETECTION OF DI-(2-AMINO-1-NAPHTHYL) HYDROGEN PHOSPHATE, A METABOLITE OF 2-NAPHTHYLAMINE IN DOGS*

By E. BOYLAND, C. H. KINDER and D. MANSON
Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, Fulham Road, London, S.W. 3 and Buckston Browne Research Farm, Downe, Kent

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Troll, Belman & Nelson (1959*a*) detected a new metabolite of 2-naphthylamine in the urine of dogs. Enzymic studies indicated that this was di-(2-amino-1-naphthyl) hydrogen phosphate. They suggested that this was a potential source of 2-amino-1-naphthol, which is thought to be the active agent in the induction of bladder tumours by 2-naphthylamine. This paper describes the synthesis of the ester and its detection, by paper chromatography, in the urine of dogs dosed with 2-naphthylamine.

EXPERIMENTAL

The dogs used were beagles dosed with 2-naphthylamine five times weekly. They were kept on a diet of canned meat and dog biscuit.

Paper chromatography. Whatman no. 1 paper was employed for descending development with the solvent systems: *a*, butan-1-ol–propan-1-ol–aq. 0·1 N-NH_3 soln. (2:1:1, by vol.); *b*, butan-1-ol–acetic acid–water (2:1:1, by vol.). For the detection of compounds on paper chromatograms the reagents used were: (1) N-HCl (or 50% acetic acid) and $NaNO_2$ (0·5%) followed by hexylresorcinol (0·5% in 2N-NaOH); (2) perchloric acid–molybdate reagent (Hanes & Isherwood, 1949); (3) 0·1 M-$K_2Cr_2O_7$–acetic acid (1:1) followed by $AgNO_3$ (0·1 M) (Knight & Young, 1958); (4) sodium 1:2-naphthaquinone-4-sulphonate (0·02 M); (5) $TiCl_3$ (15%, w/v); (6) *p*-dimethylaminocinnamaldehyde (2 g. in 100 ml. of 6N-HCl and 100 ml. of ethanol) (Harley-Mason & Archer, 1958); (7) Na_2CO_3 (10%) followed by diazotized sulphanilic acid [1·6 ml. of $NaNO_2$ (0·5%) added to 10 ml. sulphanilic acid (0·2% in N-HCl)]. A Chromatolite lamp (Hanovia Ltd.) was used as a source of u.v. light. 2-Amino-1-naphthol was detected in solution by the green derivative which is formed on the addition of conc. aq. NH_3 soln. The derivative can be extracted with benzene, in which solvent it is mauve (Liebermann & Jacobson, 1882). This test is referred to in the text as the ammonia–benzene test.

Materials. 2-Amino-1-naphthyl dihydrogen phosphate was prepared by the oxidation of 2-naphthylamine with permonophosphoric acid (Boyland & Manson, 1957*a*). The ester has R_F values 0·15 in solvent system *a* and 0·7 in solvent system *b*. On paper chromatograms the compound gives a brownish-red colour with hexylresorcinol after diazotization with HCl and $NaNO_2$, but a mauve colour after diazotization with acetic acid and $NaNO_2$.

Di-(2-nitro-1-naphthyl) hydrogen phosphate. The method of Friedmann & Seligman (1950) was used for the synthesis of this compound. Phosphoryl chloride (2·3 ml.) was added to 2-nitro-1-naphthol (10 g.) in sodium-dried benzene (100 ml.), and the mixture was heated under reflux; dry pyridine (4·2 ml.) in dry benzene was added over 20 min. and heating continued for 60 min. The solution was allowed to cool overnight and was then filtered and the filtrate evaporated to dryness *in vacuo*. The residue was dissolved in acetone (100 ml.) and water (50 ml.), heated on a steam bath for 45 min. and allowed to cool overnight, when unchanged 2-nitro-1-naphthol crystallized. This was filtered off and the filtrate evaporated *in vacuo* until a solid began to separate. After cooling, this was collected. By successive evaporation and filtration further crops were collected until they became too oily to filter. At this point the oily material was redissolved by the addition of acetone, and pyridine (5 ml.) was added. The solution was again cooled, when more crystalline material was usually obtained. The combined crops, which consisted of a

* Part 7: Boyland & Williams (1960).