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The determination of dopamine by a modification of the dihydroxyindole fluorimetric assay

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Summary

1. A simplified sensitive fluorimetric assay for dopamine based on the hydroxyindole principle is described. Oxidation of dopamine by ferricyanide and subsequent tautomerization both occur in the same, strongly alkaline, ethanolic solution in the presence of metabisulphite. The reaction is self-regulating, and since times of additions of reagents are relatively unimportant and the final fluorescence is very stable, a large number of samples can be assayed together.

2. The method was developed for assaying dopamine in N-ethanolic (50%)–HCl eluates, after purification on a strongly acidic cation exchange column as previously described (Atack & Magnusson, 1970). Portions, up to 1 ml volume, of the 3·5 ml eluate can be taken. No preliminary, time-consuming, neutralization step is required before oxidation. Fluorescence intensity shows a linear relation with concentration for 1–5,000 ng of dopamine per 1·6 ml of final solution, both in pure solutions and in column eluates.

3. Fluorescence readings from biological material are given, with evidence for their reproducibility. These, together with other data, demonstrate the relative accuracy of the 'tissue blank' and indicate that amounts of dopamine greater than 3 ng per column can confidently be detected, and amounts greater than 10 ng can be measured quantitatively.

4. Fluorescence spectra are presented for the fluorophore derived from authentic dopamine, and from suspected dopamine extracted from spinal cord of normal rats and rats treated with reserpine plus an inhibitor of synthesis. Values for the concentration of dopamine of 20 and <3 ng/g, respectively, were obtained.

Introduction

Dopamine is a widely distributed, naturally occurring compound (Holzbauer & Sharman, 1972). Its role in the central nervous system, both as a precursor to noradrenaline and as a putative neurotransmitter, has been intensively studied (Hornykiewicz, 1966; Carlsson, 1971).

Until the recent successful application of gas chromatography in combination with mass spectrometry (Karoum, Cattabeni, Costa, Ruthven & Sandler, 1972) to the determination of dopamine in nervous tissue (Koslow, Cattabeni & Costa, 1972), only fluorimetric methods possessed the required sensitivity and specificity. A compound suggested to be dopamine was first measured quantitatively in brain (Montague, 1957) by the use of a differential assay procedure based on condensation with 1,2-diaminoethane to obtain a strong fluorescence for total catecholamines

(Weil-Malherbe & Bone, 1952) together with a hydroxyindole assay in which the fluorescence contributed by dopamine was very weak (Euler & Floding, 1955). Condensation of 1,2-diaminoethane with the acetylated derivative of dopamine was used in the highly sensitive procedure developed by Laverty & Sharman (1965). A somewhat lengthy purification procedure was used to overcome the inadequate specificity of the assay, but a simplified version was subsequently described (Sharman, 1971).

The identity of dopamine in brain was established (Carlsson, Lindqvist, Magnusson & Waldeck, 1958) and its routine quantitative determination was made possible only after the development by Carlsson & Waldeck (1958) of a sensitive and specific assay based on the hydroxyindole principle. Many modifications of this basic assay were subsequently introduced, the majority to enable dopamine to be determined after the choice of different extraction and purification procedures (e.g. Drujan, Sourkes, Layne & Murphy, 1959), or because of special laboratory conditions. However, using periodate as oxidant, Anton & Sayre (1964) obtained increased sensitivity and specificity (see also Weil-Malherbe, 1968).

The dihydroxyindole assay introduced by Carlsson & Waldeck (1958) was developed for assaying dopamine after extraction and purification on a strongly acidic cation exchange column (Dowex 50), the dopamine being eluted in 12 ml N-aqueous-HCl (Bertler, Carlsson & Rosengren, 1958; Carlsson & Lindqvist, 1962). The eluate volume was significantly reduced by the incorporation of ethanol (50%) in the acid (Atack & Magnusson, 1970). For measuring dopamine in this eluate, a sensitive method was evolved from a detailed investigation of the assay described by Carlsson & Waldeck (1958). The main characteristics of their dihydroxyindole assay were (a) oxidation by iodine at pH 6·5; (b) tautomerization in strong alkali in the presence of sulphite; (c) a final acidification to 'develop' the potential fluorophore; and (d) u.v.-irradiation to accelerate this 'development'.

In the dihydroxyindole assay to be described, both oxidation, by ferricyanide, and tautomerization occur in the same highly alkaline solution. The reaction is self-regulating and times of additions of solutions are relatively unimportant. In combination with the modified Dowex 50 elution procedure, very small amounts of dopamine can be measured.

Methods

Materials

Chemicals and reagents used were dopamine (3-hydroxytyramine hydrochloride), 3-methoxytyramine hydrochloride, noradrenaline ()−)-arterenol bitartrate monohydrate), adrenaline ((−)-epinephrine bitartrate), (±)-normetanephrine hydrochloride, and 5-hydroxytryptamine (serotonin creatinine sulphate, Sigma); (±)-metanephrine hydrochloride (Winthrop); DOPA (L-β-3,4-dihydroxyphenylalanine), 3,4-dihydroxyphenylacetic acid, and tyramine hydrochloride (Fluka); 3-methoxy-L-tyrosine (Ro 8-3609, F. Hoffman-La Roche); α-methyldopamine hydrochloride (Merck, Sharp & Dohme); *N*-methyldopamine (epinine hydrochloride, Bios Labs.); histamine dihydrochloride (Merck); 6-hydroxydopamine (H 88/32), and DL-α-methyl-*p*-tyrosine-methylester hydrochloride (H 44/68, Hässle); reserpine (Serpasil, Ciba-Geigy); Dowex 50W, X-4, 200–400 mesh (Dow); NaOH (EKA); ethanol (95%, for spectroscopy) and other reagents (analytical grade) (Merck).

Water was redistilled in all-glass apparatus.

HCl—dilutions were prepared from glass-distilled 6 N HCl (a constant boiling mixture). N-ethanolic (50%)-HCl: 500 ml of 2 N HCl made up to 1 litre with 95% ethanol. 2·4 N-ethanolic (60%)-HCl: 400 ml of 6 N HCl made up to 1 litre with 95% ethanol.

Ten per cent disodium ethylene diamine tetraacetate (EDTA) (stored for less than 1 month at room temperature): 10 g were dissolved in 70 ml water, adjusted to pH 7 with 5 N NaOH, and made up to 100 ml with water.

Sodium phosphate buffer, 0·1 M, pH 6·5, in 0·1% EDTA (<1 month at 4° C): 8·83 g $NaH_2PO_4 \cdot H_2O$ + 12·88 g $Na_2HPO_4 \cdot 12H_2O$ + 10 ml 10% EDTA, made up to 1 litre with water.

Dopamine solutions were prepared in 0·01 N HCl: 500 μg base/ml stock solutions (<6 months at −20° C), 50 μg/ml (<1 month at 4° C), 10 μg/ml (<1 week at 4° C) and lower concentrations (<1 day at 4° C).

0·25% $K_3(Fe(CN)_6)$ (<1 week at 4° C); 4% EDTA (<1 month at room temperature). 3·225 N NaOH in 0·125 M $Na_2S_2O_5$ (<2 days): 15 ml 4·3 N NaOH (<1 week) + 5 ml 0·50 M $Na_2S_2O_5$ (<1 week).

Citric acid, 2·0 M, in 1·1 M phosphoric acid (<1 month): 21 g citric acid monohydrate + 5·5 ml 10 M phosphoric acid (>1 year), made up to 50 ml with water.

E-MIL disposable serological pipettes (blow out), 2 ml (H. J. Elliott), shortened to 100 mm, and the tip fitted with a small glass-wool plug.

RePettes, 2 ml and 10 ml (Jencons); silica flat-bottomed tubes, 100 mm × 12 mm (external diameter) (Thermal Syndicate); vortex laboratory mixer (Super Mixer, Lab-line Instruments); low-pressure mercury lamp, emission 254 nm (HNS, 30 watt, Osram); spectrophotofluorimeter fitted with an on-axis xenon lamp, P21 photomultiplier tube and slit arrangement no. 4 (Aminco-Bowman, American Instrument Co.); X–Y recorder (model 135 C, Moseley); and a u.v.-filter (WG $345/_2$, Schott).

Tissue extraction and column purification

Tissue was homogenized in ice-cold 0·4 N perchloric acid (5–7 ml per g of tissue, with a minimum volume of 10 ml, to which had been added 0·01 ml 5% $Na_2S_2O_5$ per ml of perchloric acid and 0·02 ml 10% EDTA per ml). The homogenate was centrifuged at about 14,000 *g* for 10 min at 0° C. The filtered supernatant was adjusted to pH 2 with 5 N K_2CO_3, usually stored at <−20° C for at most 3 days, and centrifuged at 0° C.

The Dowex 50W resin (X-4, 200–400 mesh) was thoroughly washed with 2 N NaOH in 1% EDTA and then in water until near neutral. This was followed by 2·4 N-ethanolic (60%)-HCl then 2 N HCl and finally water. Columns of resin 4·0 mm (diameter) × 85 mm long were prepared in E-MIL glass pipettes (100 mm long). The new columns were washed with 10 ml of 2·4 N-ethanolic (60%)-HCl before routine use.

In the routine washing procedure, the columns were treated with (i) 20 ml 2 N NaOH in 1% EDTA, (ii) water until near neutral, (iii) 20 ml 2 N HCl, and (iv) water until near neutral. They were then transferred to the syringe apparatus described by Bertler *et al.* (1958). After passing (a) 20 ml 0·1 M sodium phosphate buffer,

pH 6·5 (in 0·1% EDTA) (flow rate <0·5 ml/min) (length of resin in the Na^+ form was then 74–76 mm) and (b) 5 ml water, the sample was passed through the column (<0·25 ml/min; sunlight was excluded). Then, after washing the column with 15 ml water (<0·5 ml/min), 15 ml of the sodium phosphate buffer was run through (<0·5 ml/min) followed by 15 ml water. The catecholamines were eluted at 20–26° C with 10·5 ml N-aqueous-HCl (<0·25 ml/min), the last 7 ml containing noradrenaline and adrenaline, followed by 4·7 ml N-ethanolic (50%)-HCl (<0·1 ml/min), the last 3·5 ml containing the dopamine. The eluates containing dopamine could be stored for at least 1 week at −20° C. The columns were generally re-used more than 50 times.

Fluorimetric assay

Dopamine was assayed spectrophotofluorimetrically according to the schedule presented in Table 1. Three 'standards' and 3 'reagent blanks' were prepared, together with 'samples', 'internal standards' and 'unoxidized tissue blanks'. The complete procedure was carried out in silica test tubes, the contents of the tubes being thoroughly mixed on a vortex mixer after each addition and after irradiation.

TABLE 1. *Schedule for the fluorimetric determination of dopamine*

	Standard ml	Reagent blank ml	Sample ml	Internal standard ml	Tissue blank (unoxidized) ml
H_2O	—	0·05	0·05	—	0·05
4% EDTA*	0·05	0·05	0·05	0·05	0·05
0·25% $K_3(Fe(CN)_6)$†	—	—	—	—	0·05
3·225 N NaOH in 0·125 M $Na_2S_2O_5$† wait for more than 1 min	—	—	—	—	0·40
Glacial acetic acid*†	—	—	—	—	0·20
N-ethanolic (50%)-HCl	1·0	1·0	—	—	—
Eluate: (N-ethanolic (50%)-HCl)	—	—	1·0	1·0	1·0
Dopamine (base) e.g. 1 μg/ml	0·05	—	—	0·05	—
0·25% $K_3(Fe(CN)_6)$† wait for 3–6 min	0·05	0·05	0·05	0·05	—
3·225 N NaOH in 0·125 M $Na_2S_2O_5$† wait for 3–6 min	0·40	0·40	0·40	0·40	—
Glacial acetic acid*†	0·20	0·20	0·20	0·20	—

Irradiate‡ for 5 min* under a pre-warmed u.v.-lamp, and read at ca. 330/375 nm.

The final volume is 1·75 ml and the final pH is 3·8. *The EDTA can be omitted when replacing the 0·2 ml of glacial acetic acid by 0·1 ml of 2·0 M citric acid in 1·1 M phosphoric acid, the time of irradiation being increased to 7 minutes. The final volume is then 1·6 ml and the final pH is 3·6. †RePettes are normally used for these deliveries. ‡The u.v.-irradiation can be replaced by heating, e.g. in a water bath for 45 min at 72°C.

When preparing the 'unoxidized tissue blank', care was taken to ensure that the alkaline reducing mixture removed all traces of the yellow oxidant. Column eluates were brought to room temperature and thoroughly shaken before use. Portions of up to 1·0 ml of eluate were used in the assay, the portions being made up to 1·0 ml, if necessary, with pure N-ethanolic (50%)-HCl. 'Internal standards', used to indicate the degree of quenching and reproducibility of the assay, usually varied by less than ±5% for assays performed at the same time.

After the final acidification, the silica tubes, in a transparent methacrylate rack, were placed almost horizontally 15 cm beneath a u.v. lamp (peak emission 254 nm) which had been switched on 5 min beforehand to obtain a more uniform intensity

of irradiation. The complete procedure could also be performed in Pyrex glass tubes, in which case longer periods of u.v.-irradiation were required. Fluorescence was generally measured within 1 h on a spectrophotofluorimeter at activation/fluorescence wavelengths of 330/375 nm (all values given are uncorrected instrumental values). When making recordings of the spectra of the fluorescence derived from small amounts of dopamine, silica cuvettes and also a closely-matching standard were used. A Schott WG $345/_2$ u.v.-filter could be used to reduce the effect of the light scatter peak at 330 nm.

Results

For assaying dopamine in a strongly acidic (N HCl), ethanolic solution, the use of a modified dihydroxyindole fluorimetric assay is described in Table 1. The modifications eliminated the need for the time-consuming preliminary neutralization step for oxidation and consequently the associated risks of spontaneous oxidation were avoided. Times between additions of solutions could vary from 20 s to 15 min without affecting the final fluorescence. The use of RePettes enabled deliveries to be made at 5 s intervals. After u.v.-irradiation, the final fluorescence was completely stable, for several days if the tubes were placed in darkness, and for at least 4 h in a lighted laboratory, at normal room temperature (22–25° C). These factors enabled large numbers of samples to be assayed together.

The unique feature of this method is that oxidation of dopamine by ferricyanide and the subsequent tautomerization both occur in the same strongly alkaline solution in the presence of the metabisulphite reducing agent to produce a stable fluorophore.

Optimal conditions

The volume of the specified alkaline metabisulphite mixture (0·04 ml) (Table 1), could vary by more than 10% without greatly affecting the degree of oxidation and tautomerization (Figure 1a). This was so in spite of the rather critical optimal pH of 13·0–13·1 for these reactions (Figure 1b). A similar curve was obtained (Fig. 1c), where dopamine could only have been oxidized in the strongly alkaline solution containing $Na_2S_2O_5$ by the subsequently added ferricyanide. No increase in fluorescence could be gained by first oxidizing the dopamine at a lower pH and then adding the metabisulphite and alkali. Ferricyanide could be added before or after the alkaline metabisulphite mixture without affecting the amount of fluorescence produced in the reaction; but its omission resulted in only about 1% of the maximum fluorescence, arising from spontaneous oxidation.

The oxidation and tautomerization appeared to be self-regulating and occurred rapidly, as indicated by the production of maximal fluorescence when acetic acid was added 20 s after the alkaline metabisulphite. The metabisulphite reduced the ferricyanide only slowly and the reaction required at least 30 s for completion. The concentration of ferricyanide was found to be optimal with 25 ng and 5,000 ng dopamine per tube. The $Na_2S_2O_5$ could be replaced by Na_2SO_3, but no fluorescence was obtained if it was replaced by ascorbic acid or cysteine. When iodine was used as oxidant at pH 13·1, only 6% of the fluorescence obtained with the present assay was produced. The rapid, yet accurate, repetitive device, employing a forcible ejection principle for delivering the viscous alkaline metabisulphite mixture, enabled the optimal pH for oxidation to be rapidly and uniformly attained in the

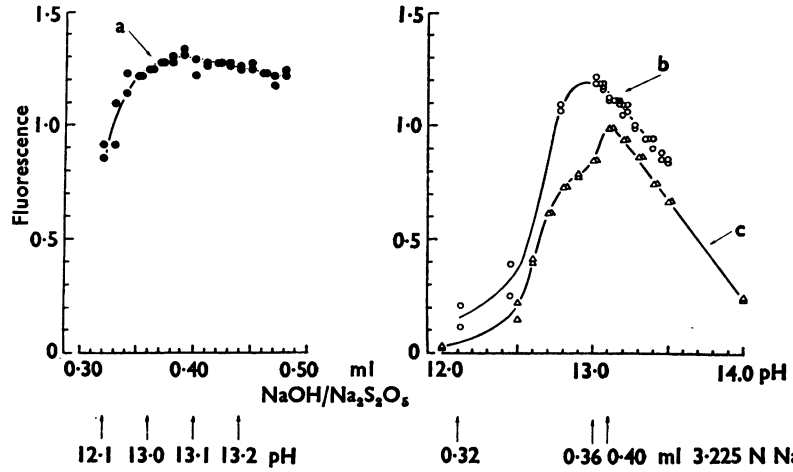

FIG. 1. The effect of variations in the volume of alkaline metabisulphite (Fig. 1a), and in the pH for oxidation and tautomerization (Fig. 1b and c), on the fluorescence derived from dopamine. (a) The volume of the alkaline metabisulphite solution added in the standard procedure (Table 1) was varied; thus, 1 ml N-ethanolic (50%)-HCl+dopamine+$K_3(Fe(CN)_6)$+different volumes of 3·225 N NaOH in 0·125 M $Na_2S_2O_5$+acetic acid+the alkaline metabisulphite to give a constant volume (1·78 ml). (b) Different alkaline pH values were obtained by varying the volume of only the NaOH added to the HCl; thus, 1·0 ml N-ethanolic (50%)-HCl+dopamine+$K_3(Fe(CN)_6)$+$Na_2S_2O_5$+(after 5 s) different volumes of 4·3 N NaOH (0·30 ml being equivalent to 0·40 ml 3·225 N NaOH) (+H_2O to give a constant volume)+acetic acid+NaOH to give a constant concentration (final volume=2·24 ml). (c) Dopamine was oxidized in solutions of different alkaline pH values, which were obtained by starting with different concentrations of NaOH containing ethanol and $Na_2S_2O_5$ (no HCl being used). Thus, 1·0 ml ethanolic (50%)-NaOH of a given concentration+$Na_2S_2O_5$+dopamine+$K_3(Fe(CN)_6)$+acetic acid+NaOH to give a constant final concentration (final volume=2·20 ml). In each series the final pH (as measured with a pH meter) was constant. The alkaline pH values indicated in (b) and (c) were based on the concentration of NaOH, which for (b) allowed for the neutralization of the HCl.

reaction mixture. Heat was generated during this step, rendering the assay insensitive to variations in room temperature.

For 'development' of the fluorophore, alternatives in the choice of final acid and source of energy are presented (see Table 1), since optimal conditions for this step may vary considerably in different laboratories. The volumes of acetic acid or citric/phosphoric acid indicated in Table 1 could vary by more than 10% without greatly affecting the final fluorescence (Figure 2a). Acetic acid has a large and increasing capacity to buffer against alkali below pH 4·4, and was more convenient to use routinely, even though approximately 15% more fluorescence could be obtained using the citric/phosphoric acid mixture. The 'development' of the fluorophore, which could only occur after a final acidification, was accelerated by the use of energy. Whilst u.v.-irradiation, tungsten lighting and heat all resulted in similar fluorescence (Fig. 3), u.v.-irradiation was most convenient, 5 min being required after the acetic acid and 7 min after the citric/phosphoric acid when using silica tubes. Longer times (e.g. 15 min after acetic acid) were required if glass tubes were used. When using heat, results obtained with Dowex 50 column eluates were more reproducible when temperatures below the boiling point of the ethanolic-HCl, e.g. 72° C, were used.

A maximal increase in fluorescence of 30% was obtained when 25–50% ethanol was incorporated in the N HCl (50% was the amount used in the Dowex 50 elution procedure—see **Methods**). The increase was only 10% if the ethanol was added

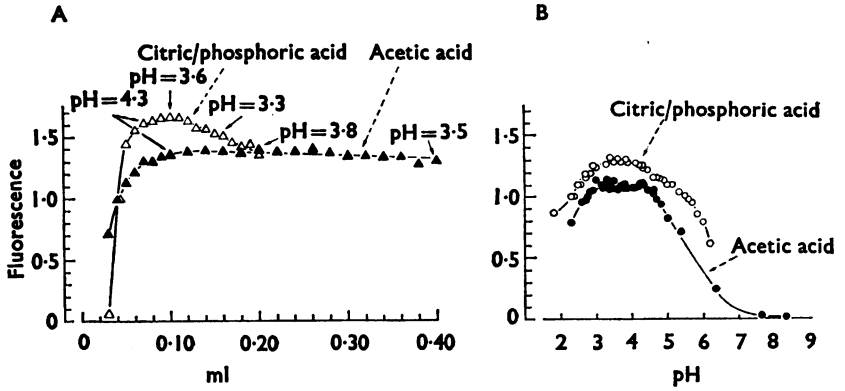

FIG. 2. The effect of variations in the volume of the final addition of acid (Fig. 2a), and in the final pH (Fig. 2b), on the fluorescence derived from dopamine. The assay procedures were as scheduled in Table 1, except that the volumes of glacial acetic acid and of 2·0 M citric acid in 1·1 M phosphoric acid were varied. The final volumes were constant only for the pH curves (3·5 and 2·2 ml for the respective acids). Activation and fluorescence wavelengths were 330/375 nm respectively.

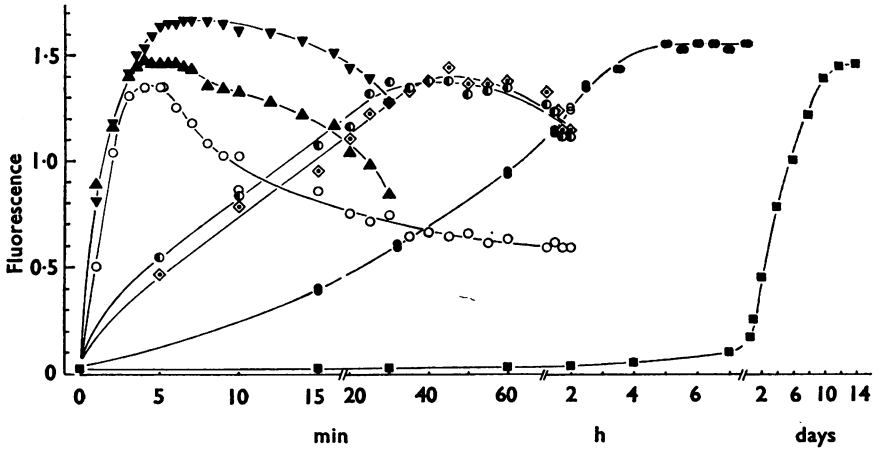

FIG. 3. The effect of light and heat on the 'development' of fluorescence of the dopamine-derived fluorophore. The fluorophore was prepared in a darkened room, but otherwise as scheduled in Table 1 using silica tubes, and, unless otherwise indicated, acetic acid. After completion, 'standards' and 'reagent blanks' were laid almost horizontal 15 cm under a u.v. lamp (peak emission 254 nm); or placed vertical in the dark or 40 cm under a 60 W tungsten lamp, either at 22° C or in a water bath at 72° or 98° C. ▼, u.v. light, citric/phosphoric acid; ▲, u.v. light, acetic acid; ○, tungsten light+heat (98° C); ◐, tungsten light+heat (72° C); ◈, darkness+heat (72° C); ●, tungsten light at 22° C; ■, darkness at 22° C.

after tautomerization. The 'reagent blank' value was dependent upon the nature of the ethanol; thus, different brands of ethanol (adjusted to 50% in N HCl) gave ratios varying between 1·2:1 (Merck, spectroscopic grade—95%) and 3:1 when the fluorescence was compared with that obtained with aqueous HCl.

Blanks

In pure solutions, accurate blanks could only be produced either as unoxidized blanks, prepared as for the 'unoxidized tissue blank' in Table 1 except that dopamine in 1 ml N-ethanolic (50%)-HCl replaced the eluate, or as 'faded blanks', prepared as for the 'standard' procedure except that the NaOH was added alone

followed after 15 min by the acid and finally the $Na_2S_2O_5$. The presence of EDTA produced lower blanks and prevented the production of a blue-green colouration which was most obvious after heating. EDTA was not necessary if citric/phosphoric acid was used.

Specificity

The degree of specificity of the assay for dopamine is indicated in Table 2. Specificity was essentially unaffected by the choice of final acid. The fluorescence derived from the compounds was measured at those wavelengths optimal for dopamine. Little increase in fluorescence was recorded when measuring the fluorescence of each compound at its own optimal wavelengths except in the case of 5-hydroxytryptamine and α-methyldopamine where fluorescence was approximately doubled. The interference by 5-hydroxytryptamine in both the 'standard' and 'tissue blank' procedures is probably caused by the strong native fluorescence in weak acid.

TABLE 2. *The relative fluorescence derived from a number of compounds in the dihydroxyindole assay for dopamine*

Compound	Percentage fluorescence at 330/375 nm in assay procedure alone	Activation fluorescence peaks nm	Concurrence in Dowex 50 procedure %	Percentage fluorescence at 330/375 nm after Dowex 50 purification
Dopamine	100	330/375	(100)	100
DOPA	67 –74	330/375	(0)	0
3-Methoxytyramine	12 –20	330/375	(20)	<4
3-Methoxytyrosine	8 –12	330/375	(0)	0
6-Hydroxydopamine	5·8 – 9·2	330/375	(<0·1)	<0·01
α-Methyldopamine	6·7 – 8·2	340/425	(98)	<8
Dihydroxyphenylacetic acid	1·4 – 1·9	330/395	(0)	0
Noradrenaline	1·1 – 1·7	330/400	(<0·1)	<0·002
Adrenaline	1·3 – 2·0	330/410	(<0·1)	<0·002
N-Methyldopamine	0·55– 0·65	330/370	(98)	<0·6
Tyramine	0·46– 0·59	335/370	(35)	<0·2
Normetanephrine	0·03– 0·06	335/380	(50)	<0·03
Metanephrine	0·04– 0·07	335/380	(70)	<0·05
5-Hydroxytryptamine	1·0 – 2·3	310/355*	(<1)	<0·02
Histamine	0·00– 0·02	none	(0)	0

*Similar to wavelengths for native fluorescence of indoles in weak acid.

The compounds were tested individually in pure solutions, both in the 'standard' and in the 'tissue blank unoxidized' procedures scheduled in Table 1. Corrected percentage fluorescences ('standard' minus 'tissue blank'), measured at wavelengths optimal for dopamine (activation 330; fluorescence 375 nm), were derived from 10 nmoles of compound (which for dopamine is equivalent to 1532 ng). Fluorescence readings for dopamine 'standards' were about 21 and for 'reagent blanks' about 0·05. 'Tissue blanks' for 6-hydroxydopamine were about 0.2, for 5-hydroxytryptamine about 0·9, and for all other compounds about 0·05. The overall percentage interference of the compounds in the determination of dopamine was calculated as the product of its maximal percentage fluorescence at 330/375 nm without previous purification and its percentage concurrence in the same eluate as dopamine after purification on Dowex 50 columns as described in **'Methods'**.

After the purification on a Dowex 50 column, overall interference by the other compounds was reduced to less than 0·6% except in the case of 3-methoxytyramine (<4%) and α-methyldopamine (<8%) (Table 2). Although α-methyldopamine, which is formed in tissues after administering α-methyltyrosine, was eluted together with dopamine, both could be estimated quantitatively using a differential assay based on measurements at different wavelengths (e.g. at 330/375 nm and 340/445 nm, giving an index of discrimination of about 14). Those compounds which are present in the same eluate as dopamine have not been found to influence the

fluorescence derived from dopamine itself, as indicated by 'internal standard' recoveries of dopamine of about 100%.

Sensitivity and reproducibility

The fluorescence derived from dopamine in pure solutions showed a linear relation with concentration for less than 1 ng to 5,000 ng per sample (Fig. 4), whichever final acid was used. Frequently, sensitivity for an assay is quoted in terms of twice the 'reagent blank', which for this assay was approximately 3·7 ng dopamine per tube. However, the high reproducibility of 'standards' and 'reagent blanks' (see Table 3) indicated that smaller amounts of dopamine could be measured. One meter unit above the 'reagent blank' (×0·001 magnification) was equivalent to less than 0·08 ng dopamine per tube, and twice the S.D. of the mean 'reagent blank' reading, i.e. 5 meter units, was thus equivalent to about 0·4 ng dopamine per tube.

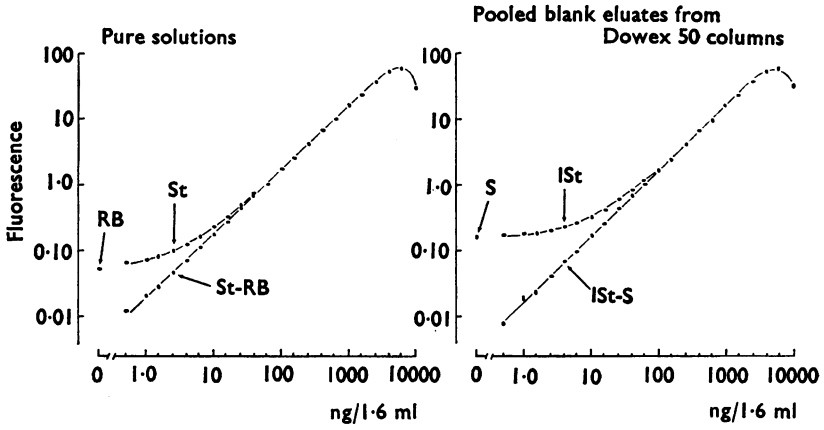

FIG. 4. Relative fluorescence derived from different concentrations of dopamine, both in pure solutions and in pooled eluates from blank Dowex 50 columns. The fluorophore was prepared as scheduled in Table 1, using citric/phosphoric acid. Two determinations were made for each concentration of dopamine. St and ISt=authentic dopamine base used in 'standards' and 'internal standards'; RB='reagent blank'; S='sample'. Both scales are logarithmic.

The assay was developed for determining dopamine in acid eluates from Dowex 50 columns. A material derived from the resin was eluted concomitantly with dopamine, which had similar peak activation/fluorescence wavelengths (322/371 nm) and which could cause a considerable increase in fluorescence when compared with the 'reagent blank'. To monitor this material, blank Dowex 50 columns were used, i.e. columns loaded with water instead of a tissue extract, but otherwise run normally. The eluate which normally contained the dopamine was collected and assayed as scheduled for the 'sample' ('purification sample') and 'unoxidized tissue blank' ('unoxidized purification blank') in Table 1.

The choice of the 'unoxidized tissue blank' procedure was based upon the behaviour of the material derived from the resin in the assay procedure. The fluorescence of this material was decreased by oxidation: thus, the 'unoxidized purification blank' reading was generally slightly higher than the 'purification sample' (3–6 min oxidation), whereas the 'faded purification blank' (15 min

oxidation—see above) was invariably lower, and its use would lead to overestimations of dopamine. The difference obtained between the 'purification sample' and 'purification blank' was termed the 'blank column error' expressed as ng dopamine per column.

Routinely, one blank column was run with every analysis of biological material. Provided the resin was thoroughly washed (see **Methods**), then during 4–5 cycles the amount of material derived from the resin was greatly reduced, as indicated by the ratio of 'purification blank' to 'reagent blank' which rapidly decreased from 15:1 to less than 4:1, and thereafter remained low. Columns could then be used 50–100 times. When this ratio was less than 4:1 small amounts of dopamine in Dowex 50 eluates could be determined more accurately, because the assay reproducibly measured the fluorescence of the material derived from the resin (Table 3), and the 'blank column error' was small. Thus, from 42 determinations obtained over several months, the mean 'blank column error' was equivalent to −0·88 ng dopamine per column (S.D. = ±2·166); a negative value being obtained because the 'unoxidized purification blank' was generally a little too high (see above). The corresponding 'internal standard' recoveries (50 ng—102±2·9%, mean±S.D., indicated no evident quenching.

TABLE 3. *Data obtained in fluorimetric determinations of dopamine in 'normal' hooded rat brains and spinal cords, with details of reproducibility of the assays*

	Acetic acid				Citric/phosphoric acid			
	Spectrophotofluorimetric readings (means)	Relative intensity (means)	Standard deviation	Coefficient of variation %	Spectrophotofluorimetric readings (means)	Relative intensity (means)	Standard deviation	Coefficient of variation %
Standards								
500 ng	63·0×0·1	=6·30	0·107	1·69	69·3×0·1	=6·93	0·093	1·34
50 ng	69·8×0·01	=0·698	0·0085	1·21	69·8×0·01	=0·698	0·0154	2·20
reagent blank	49·0×0·001	=0·0490	0·00233	4·75	49·0×0·001	=0·0490	0·00184	3·75
Blank column eluates								
'purification sample'	41·6×0·003	=0·1247	0·00193	1·55	42·5×0·003	=0·1274	0·00131	1·03
'purification blank'	42·8×0·003	=0·1283	0·00177	1·38	43·1×0·003	=0·1293	0·00155	1·20
Tissue eluates (1·0 ml of 3·5 ml)	(from 0·98 g normal rat brain extract per column)				(from 0·91 g normal rat spinal cord extract per column)			
sample tissue	85·2×0·03	=2·556	0·0247	0·97	64·5×0·003	=0·1936	0·00103	0·53
blank	44·5×0·003	=0·1335	0·00354	2·65	43·6×0·003	=0·1308	0·00118	0·90

The assays were as scheduled in TABLE 1.

All readings are the means of 10 determinations. For blank column eluates, using 8 Dowex 50 columns, 1 ml 0·9% w/v NaCl solution was substituted for tissue and the 3·5 ml eluates equivalent to the dopamine fraction were pooled. For tissue-derived eluates, 8·705 g brain from 5 rats were homogenized once in 82·4 ml perchloric acid containing EDTA and $Na_2S_2O_5$. After centrifugation and filtration, 80·5 ml of extract was recovered. The extract was divided equally between 8 columns, being the equivalent of 0·98 g of brain per column (allowance was made for the water content of the tissue (approximately 75%), since only a single extraction was made: thus

$$\frac{80{\cdot}5\times 8{\cdot}705}{(82{\cdot}4+75/100\times 8{\cdot}705)\times 8}=0{\cdot}98\text{ g.}$$

The 3·5 ml eluates were pooled and 1·0 ml portions were taken for assay. The equivalent data for spinal cords were: 8·21 g from 17 rats extracted in 82·4 ml gave a filtered extract of 78·5 ml. This was divided between 8 columns, being equivalent to 0·91 g spinal cord per column.

Fluorescence derived from dopamine added to blank column eluates also showed a linear relation with concentration for less than 1 ng to 5,000 ng per tube (Fig. 4). Values of dopamine calculated as more than 3 ng per column (upper limit of twice the standard deviation of the 'blank column error'—see above) were likely to indicate the actual presence of dopamine. These results are likely to apply also to dopamine in eluates derived from tissues, since 'tissue blanks' from whole rat brains were essentially similar to the 'purification blanks'. 'Internal standard' recoveries obtained over a period of several weeks for authentic dopamine added to eluates derived from rat brain (50 ng—$103 \pm 5{\cdot}0\%$, $n=52$; 500 ng—$99 \pm 3{\cdot}6\%$, $n=57$; mean $\pm$ S.D.) again indicated no quenching.

Thus, both 'internal standards' and blank columns were routinely measured to indicate the accuracy of the determinations of dopamine in biological material. Since the 'internal standard' recoveries were generally within $\pm 5\%$ for eluates assayed together and no quenching was evident, and since the 'blank column error' was close to zero and had a small variation when the ratio of the 'purification blank' to 'reagent blank' was less than 4:1, neither was used in the calculation of results.

Determinations of 'samples' and 'tissue blanks' from pooled eluates derived from brain or spinal cord were reproducible (Table 3). In this Table, the data have been presented to show actual values which are typical of those normally obtained when approximately 1 g of 'normal' rat brain or spinal cord was extracted and purified on a single column. From these data, dopamine concentrations can be calculated to be 688·5 ng/g and 17·0 ng/g, respectively.

Similar values for dopamine were obtained from pooled 'normal' rat brains with the present, and a second published, procedure. The pooled extracts were equally divided into 6 portions. Dopamine was determined in 3 cases after purification and fluorimetric assay as described by Carlsson & Lindqvist (1962), values of 728, 728 and 741 ng/g being obtained; and in 3 cases as described in **Methods**, values of 698, 713 and 726 ng/g being obtained. The average recoveries of dopamine added to tissue extracts was 90% in both procedures.

Finally, separate determinations of dopamine in single brains and pairs of spinal cords of rats, untreated or treated with reserpine and α-methyl-*p*-tyrosine methyl-ester, were made (Table 4). Satisfactory spectra of apparent dopamine fluorophores derived from these assays were obtained (Figure 5). The data indicated an almost complete loss of dopamine from the rat brain and spinal cord after treatment with

TABLE 4. *Dopamine concentrations in brain and spinal cord of rats treated with either reserpine and DL-α-methyl-p-tyrosine-methylester HCl, or 0·9% w/v NaCl solution*

	Brain dopamine, ng/g	Spinal Cord dopamine, ng/g
	655·9*	17·7
'Normal'	720·1	20·3*
	727·4	21·3
Reserpine	4·9*	−0·8*
+	5·1	−0·7
α-Methyl-*p*-tyrosine	5·9	0·0
(Blank column)	(0·0 ng/column)*	(−1·5 ng/column)*

*For spectra see Fig. 5. Hooded male rats, 275–345 g, were used. Six were given reserpine, 10 mg/kg i.p., at 0 h; reserpine, 5 mg/kg i.p., at 20 h; α-methyl-p-tyrosine, 250 mg/kg i.p., at 22 h; and decapitated at 24 h. Six control rats were instead given glucose twice, and 0·9% w/v NaCl solution. Each value is derived from 1 brain (1·84–1·93 g) or 2 spinal cords (1·15–1·28 g), after separate extraction, purification and assay.

drugs which release and thereby expose dopamine to metabolism by monoamine oxidase, or inhibit its synthesis, respectively. Less than 1% of the normal concentration of dopamine remained in the brain (the remaining fluorescence possibly being derived from α-methyldopamine, see Table 2, synthesized from the α-methyltyrosine) and less than 3 ng/g in the spinal cord.

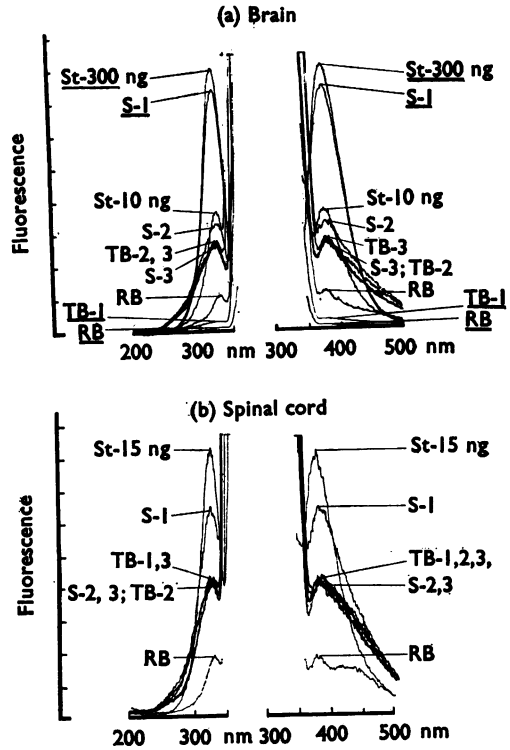

FIG. 5. Activation (left) and fluorescence spectra (right) for authentic dopamine, and for apparent dopamine extracted from brain and spinal cords of 'normal' rats and rats treated with reserpine plus α-methyl-*p*-tyrosine. St='standard': ng of authentic dopamine base/tube; RB=reagent blank; S=sample; and TB='unoxidized tissue blank'. -1='normal', -2=reserpine+α-methyl-*p*-tyrosine and -3=blank Dowex 50 column (those underlined were recorded on a 10 times lower sensitivity setting). The fluorophores were prepared as scheduled in Table 1, acetic acid being used for (a) and citric/phosphoric acid for (b). The spectra were recorded with the fluorescence set to 375 nm (left) and the activation wave-length set to 330 nm (right) (optima on the instrument used), and a Schott WG 345/2 u.v.-filter was used for (b).

Discussion

Practical considerations

A modified dihydroxyindole assay is described. Oxidation of dopamine by ferricyanide was shown to occur under the same strongly alkaline conditions as for tautomerization in the presence of metabisulphite. Advantages of this assay compared with other hydroxyindole assays for the determination of dopamine are as follows. The period of exposure to the oxidizing agent is relatively unimportant since the oxidation is self-regulating and the final fluorescence is completely stable for at least 4 h (cf. Anton & Sayre, 1964). When the dopamine is initially present in a strongly acidic solution (e.g. N HCl), no time-consuming neutralization step, with concomitant risks of spontaneous oxidation, is required prior to the controlled

oxidation (cf. Carlsson & Waldeck, 1958). Consequently, a large number of samples can be assayed together. The ratio of the volume of eluate used, to the final assay volume, is large (1:1·6). Finally, in contrast to the iodine oxidation technique, we have not found this assay to be sensitive to environmental conditions.

The sensitivity of this assay in pure solutions in terms of twice the 'reagent blank' (2·3 ng/ml of final solution), appears to lie between those of Anton & Sayre (1964) (5·1 ng/ml) and Sharman (1971) (1·9 ng/ml), although the reproducibility of the 'reagent blank' enables smaller amounts to be determined. To assess accurately the sensitivity of an assay procedure, the mean instrumental readings ±S.D. for the 'reagent blank' and a 'standard' (presented for this assay in Table 3) are useful.

The 'blank column error' defined as the difference between the 'purification sample' and the 'purification blank', and its variability for a specified combination of assay and purification procedures, is one indication of the minimum amount of a compound which can be detected with the method. This 'error' has not been evaluated for most methods, including assays in combination with alumina column purification (where it may be high; see Weil-Malherbe, 1968). The behaviour of the interfering material from the purification procedure in the 'sample' and 'tissue blank' is in agreement with the findings of Häggendal (1962). The use of the 'unoxidized tissue blank' (Table 1) is preferred to a 'faded tissue blank', since the latter is more likely to result in an over-estimation of dopamine in tissues.

The 'development' of the fluorescence of the hydroxyindole formed from dopamine, which in alkaline solution is only weakly fluorescent (in contrast to that of noradrenaline e.g. Häggendal, 1963), can only occur after acidification. This 'development' can then be accelerated by the introduction of energy, but the chemical changes involved are unknown. Considerable discrepancies exist in the conditions required to accelerate this 'development' in both the iodine technique (Carlsson & Waldeck, 1958; Laverty & Taylor, 1968; Welch & Welch, 1969) and the periodate technique (Anton & Sayre, 1964; Weil-Melherbe, 1968). The final acidification also serves to increase specificity by inducing a hypsochromic wavelength shift which is considerably greater for those phenethylamine derivatives which do not bear a β-hydroxyl group in the side chain (Laverty & Taylor, 1968). The use of ethanol and of a citric/phosphoric acid mixture to induce additional increases in fluorescence was described earlier by Anton & Sayre (1964).

The specificity of the present assay and of other commonly used dihydroxyindole assays for dopamine (Carlsson & Waldeck, 1958; Anton & Sayre, 1964) is relatively high, although a number of compounds do interfere, notably DOPA and 3-methoxytyramine. By prior purification on a Dowex 50 column, the interference by most compounds can be either eliminated or greatly reduced. Thus, the complete separation of dopamine from DOPA is very useful in studies where high concentrations of DOPA are induced, e.g. after administration of DOPA or inhibitors of the aromatic amino acid decarboxylase (Carlsson, Davis, Kehr, Lindqvist & Atack, 1972). Although 3-methoxytyramine is incompletely separated from dopamine in the present Dowex 50 procedure, its interference in the present assay (12–20%) is considerably less than after iodine oxidation (48%—personal observation of the method by Carlsson & Lindqvist, 1962). The overall interference by 3-methoxytyramine of less than 4% is acceptable for most purposes, since the ratio of dopamine to its major metabolite is normally high, and is rarely reversed

even after monoamine oxidase inhibition (Carlsson & Waldeck, 1964; Guldberg, Sharman & Tegerdine, 1971). Dopamine can be completely separated from noradrenaline and adrenaline on Dowex 50 columns (Bertler *et al.*, 1958). This can be valuable before hydroxyindole assays (see Table 2; also Carlsson & Waldeck, 1958), and was essential in the simplified and sensitive procedure for dopamine described by Sharman (1971) to avoid potential interference by catechols in the ethylene diamine condensate assay. Non-catechols, e.g. 3-methoxytyramine, do not interfere in this latter assay.

Of the other procedures most commonly used for purifying dopamine prior to its assay, solvent extraction (e.g. Welch & Welch, 1969) results in the least removal of interfering compounds. Further purification is generally used, e.g. alumina (e.g. Chang, 1964) which eliminates interference by 3-methoxytyramine but not catecholamines and DOPA. The greater specificity of the dihydroxyindole assay using periodate (Anton & Sayre, 1964) can be made use of following these purification procedures (cf. Chang, 1964). However, differential assays (Anton & Sayre, 1964) for estimating small amounts of dopamine in the presence of DOPA is less satisfactory (cf. Weil-Malherbe, 1968).

By using ethanolic-HCl for eluting dopamine from Dowex 50 columns, the concentrating effect obtained for dopamine in the above three types of purification procedure is now similar. The increased sensitivity and the specificity achieved by measuring dopamine with the present, more tolerant, dihydroxyindole assay further increases the usefulness of the Dowex 50 single-column procedure. Other putative transmitters and related compounds can also be eluted separately and assayed fluorimetrically (see Atack & Magnusson, 1970; Lindqvist, 1971; Kehr, Carlson & Lindqvist, 1972; Atack & Lindqvist, 1973).

Theoretical considerations

In the present assay, oxidation of dopamine by ferricyanide in a strongly alkaline solution in the presence of metabisulphite is probably complete. At a pH of not less than 7, Harrison, Whisler & Hill (1968) showed that dopamine was completely oxidized by ferricyanide, almost instantaneously, as easily as were noradrenaline and adrenaline. Previously, stronger oxidizing agents such as iodine, manganese dioxide or periodate have been used for oxidizing dopamine (Carlsson & Waldeck, 1958; Uuspää, 1963; Anton & Sayre, 1964), although ferricyanide is widely used for oxidizing noradrenaline and adrenaline.

Probably in alkaline solutions the redox potentials of the system are such that metabisulphite is unable to prevent oxidation of catecholamines by ferricyanide to quinone derivatives, but in the case of dopamine prevents further oxidation to melanin products (see Dulière & Raper, 1930). Under such alkaline conditions, tautomerization can occur with the formation of a potentially fluorescent dihydroxyindole. In other fluorimetric assays, ferricyanide is frequently used in conjunction with the reducing agents ascorbic acid (e.g. Euler & Floding, 1955) or cysteine (e.g. Guldberg *et al.*, 1971). However, in the present assay the redox potentials established by such combinations were unsuitable; in these cases the yellow ferricyanide colour disappeared almost instantaneously, in contrast to its slow disappearance when using sulphites. Furthermore, the use of iodine as oxidant was unsatisfactory; its brown colour disappeared almost instantaneously with any of the three reducing agents. Previous results suggest that iodine may have its own specific pH require-

ments for the oxidation of different catecholamines (see Laverty & Taylor, 1968).

The development of this method is interesting in view of the early history of hydroxyindole assays (see Euler, 1959). In the early assays for adrenaline, an unstable fluorescence was formed after oxidation by dissolved oxygen in strongly alkaline solutions. To obtain greater stability and reproducibility, oxidation was performed at a more neutral pH with, for example, ferricyanide, and ascorbic acid was incorporated in the NaOH to prevent further oxidation during the subsequent tautomerization step (Ehrlén, 1948). In this form, the trihydroxyindole assay was investigated by Carlsson & Waldeck (1958) and adapted for the assay of dopamine. Considering that the present assay for dopamine bears resemblances in design to some of the earlier unsatisfactory trihydroxyindole assays and because of possible reservations in accepting a method which depends upon a specific pH being achieved by mixing strong alkali with strong acid in an unbuffered solution, and because materials are eluted by acid from strongly acidic cation exchange columns which can interfere in fluorimetric assays, considerable attention was paid to demonstrating the tolerance in optimal conditions and to the high reproducibility and accuracy of the assay.

Biological considerations

Dopamine values of 700 ng/g for brain and 20 ng/g for spinal cord of 'normal' rats were in good agreement with values of 680 ng/g and 20 ng/g, respectively, obtained by Anton & Sayre (1964). The method has been successfully applied to the determination of dopamine in peripheral tissues, including sheep thyroid gland (Atack, Ericson & Melander, 1972) and rat adrenals (Snider & Carlsson, 1972).

The presence of dopamine, 20 ng/g, in 'normal' rat spinal cord was further supported by satisfactory activation and fluorescence spectra, and by the fact that the fluorescence was reduced to less than 1% after treatment with reserpine plus an inhibitor of catecholamine synthesis.

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