# STUDIES ON CATECHOLAMINES AND 5-HYDROXYTRYPTAMINE AND THE SIGNIFICANCE OF THEIR METABOLITES IN ANIMAL TISSUES AND BODY FLUIDS

by

D. F. SHARMAN

B.Sc., Ph.D.,

University of Edinburgh

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Summary

The papers presented in this thesis describe the development of methods for the estimation of adrenaline, noradrenaline, dopamine, 5-hydroxytryptamine and some of their metabolites and the application of such estimations to some problems of biological interest. The major part of the thesis is concerned with the metabolism of these amines in the mammalian central nervous system. The papers are presented in three groups.

The first group is made up of papers in which the estimation of 5-hydroxytryptamine (5-HT) or its metabolite 5-hydroxy-indol-3-yl acetic acid (5-HIAA) was measured.

1. On the question of the occurrence and metabolism of 5-hydroxy-tryptamine and related indole compounds in mammalian semen. By T. Mann, R. F. Seamark and D. F. Sharman. Br. J. Pharmac. Chemother. 17, 208 - 217, 1961.

In this paper it was shown conclusively that the semen of man, bull, boar, ram and dog contains little or no 5-hydroxytryptamine.

2. Drug-induced changes in the concentration of 5-OR indolyl compounds in cerebrospinal fluid and caudate nucleus. By G. W. Ashcroft and D. F. Sharman. Br. J. Pharmac. Chemother. 19, 153 - 160, 1962.

Because of an earlier observation by Ashcroft and Sharman (Nature, Lond., 186, 1050 - 1051, 1960) that the cerebrospinal fluid of depressed patients contained a lower concentration of 5-hydroxyindolyl compounds than normal, the effect of reserpine, a drug known to reduce the concentration of 5-hydroxytryptamine in the brain, on the concentration of such compounds in the cerebrospinal fluid of the dog was examined. It was found that the concentration of 5-hydroxyindolyl compounds in the cerebrospinal fluid was increased after reserpine.

3. The effect of α-methyldopa on the metabolism of 5-hydroxy-tryptamine in rat brain. By D. F. Sharman and S. E. Smith. J. Neurochem. 9, 403 - 406, 1962.

In this paper, the concentration of 5-HIAA in the brain was used as an index of the rate at which 5-HT was released in this tissue after α-methyldopa, an inhibitor of the formation of 5-HT, was given to rats.

4. The action of 2-aminotetralin (β-tetrahydronaphthylamine) on the metabolism of 5-hydroxytryptamine in the brain of the mouse. By D. Robinson and D. F. Sharman. Br. J. Pharmac. Chemother. 29, 335 - 341, 1967.

2-Aminotetralin causes a reduction in the concentration of 5-hydroxyindol-3-ylacetic acid in the brain of the mouse (paper 12). The possible causes of this effect were examined.

The second group consists mainly of papers which describe the development of methods of estimating dopamine and its acid metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and 4-hydroxy-3-methoxyphenyl acetic acid (homovanillic acid; HVA), their application to several problems, chiefly to a study of the rate of utilisation of dopamine in the brain and also the effect of drugs on the metabolism of this amine.

5. Chemical and physiological changes produced by arterial infusion of dihydroxyphenylalanine into one cerebral hemisphere of the cat. By R. Dagirmanjian, R. Laverty, P. Mantegazzini, D. F. Sharman and M. Vogt. J. Neurochem. 10, 177 - 182, 1963.

The infusion of 3,4-dihydroxyphenylalanine (DOPA) into one carotid artery of the cat can cause arousal of the brain on the side of the infusion. It was shown that this unilateral arousal is accompanied by an increase in the concentration of dopamine in the caudate nucleus, hypothalamus and midbrain reticular formation on the same side of the brain.

6. The subcellular localisation of dopamine and acetylcholine in the dog caudate nucleus. By R. Laverty, I. A. Michaelson, D.F. Sharman and V. P. Whittaker. Br. J. Pharmac. Chemother. 21, 482 - 490, 1963.

7. Localisation of acetylcholine, 5-hydroxytryptamine and noradrenaline within subcellular particles derived from guinea-pig subcortical brain tissue. By I. A. Michaelson, V. P. Whittaker, R. Laverty and D. F. Sharman. Biochem. Pharmacol. 12, 1450 - 1453, 1963.

These two papers describe the estimation of noradrenaline, dopamine, 5-hydroxytryptamine and acetylcholine in particles obtained by subcellular fractionation of brain tissues. The first shows that dopamine is associated with a particle that is similar to, but distinguishable from, that with which acetylcholine is associated. The second paper demonstrates that the storage sites within subcellular particles for acetylcholine and those for noradrenaline and 5-hydroxytryptamine are different.

8. A fluorimetric method for the estimation of 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid) and its identification in brain tissue. By D. F. Sharman. Br. J. Pharmac. Chemother. 20, 204 - 213, 1963.

9. The estimation of small quantities of 3,4-dihydroxyphenylethylamine in tissues. By R. Laverty and D. F. Sharman. Br. J. Pharmac. Chemother. 24, 538 - 548, 1965.

10. Modification by drugs of the metabolism of 3,4-dihydroxyphenylethylamine, noradrenaline and 5-hydroxytryptamine in the brain. By R. Laverty and D. F. Sharman. Br. J. Pharmac. Chemother. 24, 759 - 772, 1965.

11. The effect of drugs on the homovanillic acid content of the corpus striatum of some rodents. By A. V. Juorio, D. F. Sharman and T. Trajkov. Br. J. Pharmac. Chemother. 26, 385 - 392, 1966.

12. Changes in the metabolism of 3,4-dihydroxyphenylethylamine (dopamine) in the striatum of the mouse induced by drugs. By D. F. Sharman, Br. J. Pharmac. Chemother. 28, 153 - 163, 1966.

13. A discussion of the modes of action of drugs which increase the concentration of 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid) in the striatum of the mouse. By D. F. Sharman. Br. J. Pharmac. Chemother. 30, 620 - 626, 1967.

14. Homovanillic acid and dihydroxyphenylacetic acid in the striatum of monkeys with brain lesions. By D. F. Sharman, L. J. Poirier, G. F. Murphy and T. L. Sourkes. Can. J. Physiol. Pharmacol. 45, 57 - 62, 1967.

15. Release by tubocurarine of dopamine and homovanillic acid from the superfused caudate nucleus. By P. J. Portig, D. F. Sharman and Marthe Vogt. J. Physiol. Lond. 194, 565 - 572, 1968.

16. The effect of tropolone on the formation of 3,4-dihydroxyphenylacetic acid and 4-hydroxy-3-methoxyphenylacetic acid in the brain of the mouse. By G. F. Murphy, D. Robinson and D. F. Sharman. Br. J. Pharmac. Chemother. 36, 107 - 115, 1969.

17. Turnover of amines using probenecid to block the egress of metabolites. By D. F. Sharman. Metabolism of brain amines. Edited by G. Hooper, Macmillan, London. pp. 34 - 37, 1969.

These papers form the main part of the thesis and attempt to relate the concentration of HVA in the central nervous system to the rate at which dopamine is utilised in this tissue. The locus of the metabolism of dopamine in the central nervous system is discussed.

The papers in the third group are concerned with noradrenaline or its glycol metabolites.

18. Noradrenaline content in the heart and spleen of the mouse under normal conditions and after administration of some drugs. By D. F. Sharman, S. Vanov and Marthe Vogt. Br. J. Pharmac. Chemother. 19, 527 - 533, 1962.

This study was made to investigate a report of what appeared to be unusual behaviour of the tissue catecholamines in the mouse. The method used to estimate the noradrenaline was designed to incorporate as many controls as was possible to ensure that correct estimates were obtained. The earlier report could not be confirmed.

19. Iontophoretic release of adrenaline noradrenaline and 5-hydroxytryptamine from micropipettes. By K. Krnjević, R. Laverty and D. F. Sharman. Br. J. Pharmac. Chemother. 20, 491 - 496, 1963.

Fluorimetric methods were used to measure the relation between the release of these amines and the electrical charge applied to micropipettes used for the iontophoretic application of drugs to single neurones.

20. The action of 2,4,5-trihydroxyphenylethylamine on the storage and release of noradrenaline. By R. Laverty, D. F. Sharman and Marthe Vogt. Br. J. Pharmac. Chemother. 24, 549 - 560, 1965.

2,4,5-Trihydroxyphenylethylamine causes a rapid and long lasting depletion of noradrenaline from the mouse heart. A new method was developed for the estimation of the former amine and was used to show that it did not persist in the tissue or was tightly bound in the tissue.

21. The noradrenaline content of the caudate nucleus of the rabbit. By D. F. Sharman and Marthe Vogt. J. Neurochem. 12, 62, 1965.

This short paper illustrates a frequently reported erroneous result when a commonly used fluorimetric method for the estimation of noradrenaline is applied to those brain tissues which contain very little of this amine.

22. Gas chromatographic evidence for the presence of glycol metabolites of catecholamines in brain tissue. By D. F. Sharman. J. Physiol. Lond. 200, 33 - 35P, 1969.

23. Glycol metabolites of noradrenaline in brain tissue.

By D. F. Sharman. Accepted for publication by Br. J. Pharmac. Chemother. 1969.

The application of gas liquid chromatography and electron capture detection to the estimation of glycol metabolites of noradrenaline in the brain is described. The possibility of using a similar technique for the estimation of noradrenaline and normetanephrine is discussed.

# ON THE QUESTION OF THE OCCURRENCE AND METABOLISM OF 5-HYDROXYTRYPTAMINE AND RELATED INDOLE COMPOUNDS IN MAMMALIAN SEMEN

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T. MANN, R. F. SEAMARK and D. F. SHARMAN

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# ON THE QUESTION OF THE OCCURRENCE AND METABOLISM OF 5-HYDROXYTRYPTAMINE AND RELATED INDOLE COMPOUNDS IN MAMMALIAN SEMEN

BY

T. MANN, R. F. SEAMARK AND D. F. SHARMAN

*From the A.R.C. Unit of Reproductive Physiology and Biochemistry, School of Veterinary Medicine, and Molteno Institute, University of Cambridge, and A.R.C. Institute of Animal Physiology, Babraham, Cambridge*

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The content of 5-hydroxytryptamine was examined in the semen of man, bull, ram, boar and dog. Spectrophotofluorimetric and chromatographic analysis of extracts prepared by several methods has shown that the semen contains only minute quantities of 5-hydroxytryptamine, if any. One cannot attribute to 5-hydroxytryptamine the uterus-stimulating effect which the human seminal plasma is known to exert. Tryptophan, although present in the seminal plasma, is not converted to 5-hydroxytryptamine on incubation of semen. 5-Hydroxytryptophan is oxidatively deaminated, but not decarboxylated, by spermatozoa. 5-Hydroxytryptamine itself, like tryptamine and tyramine, is not deaminated by either whole semen or washed suspensions of ram spermatozoa.

Among the pharmacodynamic effects of the mammalian seminal plasma is a strong stimulation of smooth-muscle organs such as the uterus and the intestine. This oxytocic activity of semen was investigated in the past by many authors, including Kurzrok & Lieb (1930), Cockrill, Miller & Kurzrok (1935), Goldblatt (1933, 1935a, b), von Euler (1934a, b ; 1935, 1936, 1939, 1949), Vandelli (1943), Asplund (1947a, b), Karlson (1949) and Bergström (1949) ; the general conclusion reached was that the uterine-stimulating property of semen, particularly in man, is probably due to the combined action of several constituents of the seminal plasma, such as choline, prostaglandin and histamine. The early literature has been reviewed in some detail by Mann (1954). Seminal prostaglandin has since been purified and is now believed to be produced, at least in man and ram, by the seminal vesicles and not by the prostate gland (Eliasson, 1959). Two crystalline compounds, both possessing the properties of nitrogen-free unsaturated fatty-acids, have been separated from prostaglandin preparations of vesicular origin ; one of these, " prostaglandin E," was shown to exert, in addition to the smooth-muscle stimulating action, a depressant effect on blood pressure of the rabbit, while the other, " prostaglandin F," was found to be devoid of action on blood pressure (Bergström, Eliasson, Euler & Sjövall, 1959 ; Bergström, Duner, Euler, Pernow & Sjövall, 1959).

Several authors have also considered the possibility that the " oxytocic activity " of semen is due, at least partly, to 5-hydroxytryptamine, which is known to induce

strong uterine contractions, either when applied to the isolated uterus of the rat (Erspamer, 1952) or injected into a living dog (Abrahams & Pickford, 1956). The evidence, however, for the presence of this substance in mammalian semen has so far been confusing and contradictory. Thus, whereas according to Katsh (1959) human seminal plasma contains as much as 135 $\mu$g/ml., Hawker, Roberts & Walmsley (1960) could not find 5-hydroxytryptamine either in human or in ram semen.

On the other hand, a recent investigation of the secretory function of male accessory organs in the spiny dogfish, *Squalus acanthias,* has revealed the surprising fact that, in this viviparous elasmobranch fish, one of the accessory organs, the so-called clasper siphon, produces a secretion which is extremely rich in 5-hydroxytryptamine (Mann, 1960). The presence of as much as 7% of 5-hydroxytryptamine in a secretion which forms part of the seminal plasma in the spiny dogfish suggests that in this animal 5-hydroxytryptamine may be involved in the process of reproduction, either by facilitating the ejaculation of semen in the male or perhaps by eliciting contractions of the reproductive tract in the female and thus aiding sperm ascent in the oviducts. Another recent observation relevant to the function of 5-hydroxytryptamine in reproduction concerns certain insects. It was shown that the secretion of the opaque accessory gland in the male of *Rhodnius prolixus,* as well as the secretion of similar glands, the utriculi majores, in the cockroach *Periplaneta americana,* contain a substance which causes contractions of the insects' oviducts. The active principle is destroyed by monoamine oxidase and *o*-diphenol oxidase, and is probably an *o*-dihydroxyindolalkylamine related to 5-hydroxytryptamine (Davey, 1960).

We have attempted to clarify the question concerning the presence of 5-hydroxytryptamine and related compounds in mammalian semen, and to provide more information on the relation of 5-hydroxytryptamine to the "oxytocic activity" of semen.

## METHODS

*Semen.* Semen from rams, bulls and boars was collected by means of the artificial vagina. Dog semen provided by Dr D. Bartlett was collected by the massage technique. Human semen from donors at the Fertility Clinic was made available by Dr H. A. Davidson. The semen samples intended for determination of 5-hydroxytryptamine were kept in solid carbon dioxide until deproteinized. In addition to whole boar semen, analyses of 5-hydroxytryptamine were also carried out on the seminal gel and the epididymal seminal plasma of this species. For measurements of respiration, fresh ram semen was used, either whole or after separation into spermatozoa and seminal plasma.

*Spectrophotofluorimetric determination of 5-hydroxytryptamine.* This was carried out in the Aminco-Bowman spectrophotofluorimeter on extracts prepared by one of the following three procedures: (1) The sample was deproteinized with zinc sulphate and sodium hydroxide; deproteinization was usually carried out by adding to the sample 1 vol. water, followed by 1 vol. of 10% zinc sulphate ($ZnSO_4.H_2O$) and 0.2 vol. of 10% sodium hydroxide; on occasions when the centrifuged extract was not completely clear, small additional amounts of the deproteinizing agents had to be added; to 2 ml. of the protein-free filtrate was added 1 ml. concentrated hydrochloric acid containing ascorbic acid (0.1 mg/1 ml.); the solution was placed in a quartz cuvette for spectrophotofluorimetric analysis. (2) The sample was extracted by the method of Bogdanski, Pletscher, Brodie & Udenfriend (1956). (3) The sample was treated with 4 vol. acetone and the resultant precipitate and supernatant solution processed further as described by Katsh (1959).

*Chromatographic identification of 5-hydroxytryptamine and related indole compounds.* Both descending and ascending one- and two-dimensional paper partition chromatography was used. The solvent systems, (i) butanol + acetic acid, (ii) isopropanol + ammonia, (iii) aqueous potassium chloride, and (iv) butanol + pyridine, were used as recommended by Jepson (1960). The developed chromatograms were dried at 25° C in an air oven, all fluorescent areas noted, and an ultra-violet contact photograph taken, using a Hanovia " Chromatolite " lamp and Kodak Duostat 13 reflex paper. The following location reagents, used either singly or in sequence, were applied: acid ninhydrin, ninhydrin pyridine, Ehrlich's reagent, diazotized sulphanilic acid (Jepson, 1960), 5 N hydrochloric acid (Sharman, 1960), and potassium dichromate formaldehyde (Shepherd & West, 1953).

*Metabolic studies.* Oxygen uptake was determined in Barcroft differential manometers filled with air, at 37° C. Ammonia formation was followed by treating the samples with a saturated solution of sodium borate and subjecting the mixtures to steam-distillation *in vacuo,* at a temperature not exceeding 25° C, in the apparatus of Parnas & Heller (1924). Monoamine oxidase activity was determined by measuring $O_2$ uptake and ammonia formation as well as by the colorimetric method of Green & Haughton (1961).

*Chemicals.* The chemicals were of the analytical or microanalytical reagent grade: 5-Hydroxytryptamine creatinine sulphate (Sigma Chem. Co.; May & Baker); dl-tryptophan (Light & Co.); 5-hydroxytryptophan (Roche); tryptamine and tyramine hydrochlorides (British Drug Houses).

## RESULTS

### *Spectrophotofluorimetric observations*

*Experiment no. 1*

This was carried out on a series of extracts prepared from human, ram, bull and boar semen by deproteinization with zinc hydroxide, and the supernatant then rendered 3 N with respect to hydrochloric acid. When these extracts were examined in the spectrophotofluorimeter using a Chance OY 4 filter in the fluorescent light path, a distinct fluorescence was observed with an activation maximum at 295 m$\mu$ and a fluorescence maximum at 525 m$\mu$. This fluorescence maximum, however, was shown to be an artefact, due to the transmission characteristics of the filter, since, when the determinations were repeated with the filter omitted, the wavelengths for maximal activation and fluorescence of the seminal extracts were 295 m$\mu$ and 430 m$\mu$, respectively. On the instrument used, 5-hydroxytryptamine, in common with other 5-hydroxyindolyl derivatives, showed a fluorescence in 3 N HCl, characterized by an activation maximum at 285 m$\mu$ and a fluorescence maximum at 540 m$\mu$. Thus the seminal constituent which fluoresced with a maximum at 430 m$\mu$ could not be identical with 5-hydroxytryptamine, even though its activation peak of 295 m$\mu$ is close to that of 5-hydroxytryptamine.

In the light of this observation, subsequent fluorimetric analyses of the seminal extracts were made using an activation wavelength of 285 m$\mu$ and a fluorescence wavelength of 540 m$\mu$, and calibrating each time the observed fluorescence against one produced by a standard solution of pure 5-hydroxytryptamine. When this was done the following results were obtained, the 5-hydroxytryptamine content being expressed in ng/2 ml. extract: three different samples of human semen, 0 to 5; ram semen, 0; ram seminal plasma, 0; two different samples of bull semen, 0 and 60; boar semen (separated from the gel), 250; boar seminal gel, 0; boar epididymal seminal plasma, 0.

As can be seen, the fluorescence values were of an exceedingly low order in all species. Moreover, these values represent not only the maximum fluorescence attributable to 5-hydroxytryptamine, but include, in all probability, the fluorescence due to some other substances which are present in semen.

*Experiment no. 2*

For the detection of trace amounts of 5-hydroxytryptamine in semen, an experiment was carried out on two 1.5 ml. samples of human semen, to one of which 5 μg 5-hydroxytryptamine was added before deproteinization. Both samples were extracted with butanol according to Bogdanski *et al.* (1956), and the purified and acidified extracts used to determine the activation scans with fluorescence at 540 mμ, as well as the fluorescence scans with activation at 280 mμ. The results of these determinations are recorded in Fig. 1, which shows the characteristic fluorescence

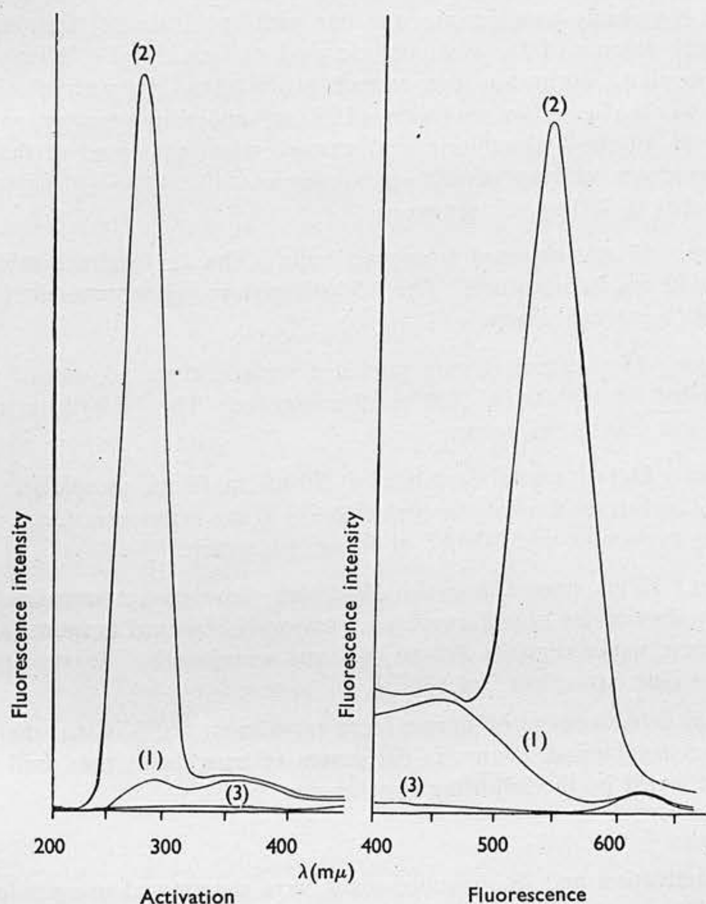


Fig. 1. Activation and fluorescence spectra of extracts from human semen: (1) extract from 1·5 ml. human semen; (2) extract from 1·5 ml. human semen to which 5 μg 5-hydroxytryptamine was added; and (3) reagent blank.

due to added 5-hydroxytryptamine and the lack of fluorescence in the sample without 5-hydroxytryptamine. These results clearly indicate that the content of 5-hydroxytryptamine in human semen is either negligible or nil.

*Experiment no. 3*

The fluorimetric analyses of human, bull, boar and ram semen were repeated, using larger quantities of semen and the extraction procedure of Bogdanski *et al.* (1956) which allows the preparation of more concentrated extracts. In addition, dog semen was examined by the same method. In each instance two measurements were taken: (i) of the wavelengths of maximal activation and fluorescence of the seminal extracts; and (ii) of the " 5-hydroxytryptamine equivalent" of any fluorescence present, determined at the activation wavelength of 285 mμ, and the fluorescence wavelength of 540 mμ. The results were as follows.

*Human semen.* 9 ml. representing pooled ejaculates from four donors; two normal, one completely azoospermic, and one with sperm density less than 10,000 cells/μl.; final volume of the hydrochloric acid extract, 5 ml. When examined in the spectrophotofluorimeter, the extract exhibited a characteristically strong fluorescence with an activation maximum of 295 mμ and a fluorescence maximum at 430 mμ; 2 ml. of the hydrochloric acid extract, when examined at the 285 and 540 mμ wavelength, corresponded to a content of 540 ng " 5-hydroxytryptamine equivalent," that is, 150 ng/ml. semen.

*Bull semen.* 13 ml. obtained from two bulls. The acid extract exhibited the typical 295/430 mμ fluorescence. The " 5-hydroxytryptamine equivalent" content was 1,000 ng (1 μg)/ml. semen.

*Ram semen.* 15 ml. representing pooled ejaculates from ten rams. The acid extract exhibited the typical 295/430 mμ fluorescence. The " 5-hydroxytryptamine equivalent" was 500 ng/ml. semen.

*Boar semen.* Out of a total ejaculate of 270 ml., a 15 ml. sample was used for extraction. The extract showed the typical 295/430 mμ fluorescence, and contained a " 5-hydroxytryptamine equivalent" of 50 ng/ml. semen.

*Dog semen.* 15 ml. representing two ejaculates. The extract prepared from dog semen clearly showed the blue fluorescence previously observed in human, bull, ram and boar semen, namely, at the 295 and 430 mμ wavelengths. It contained a " 5-hydroxytryptamine equivalent" of 150 ng/ml. semen.

The general conclusion to be drawn from experiment no. 3 is that there is very little 5-hydroxytryptamine, if any, in the semen of man, boar, ram, bull and dog. This was confirmed by the following experiment.

*Experiment no. 4*

Both the activation and fluorescence scans were determined using side by side the hydrochloric acid extracts obtained from human and canine semen, and a similarly prepared extract from the nucleus caudatus of the dog brain, of which the 5-hydroxytryptamine content had previously been determined and found to be

0.3 μg/g tissue. The results of this experiment are recorded in Fig. 2. In the left part of this figure the activation scan of the extract from dog semen is compared with that obtained from the nucleus caudatus. There is only a small difference in the maximum activation wavelength (295 mμ and 285 mμ, respectively). In the right part of Fig. 2, the fluorescence scan of human semen is contrasted with that of the dog nucleus caudatus; here it is obvious that the peak characteristic of 5-hydroxytryptamine (540 mμ) is entirely absent in the semen.

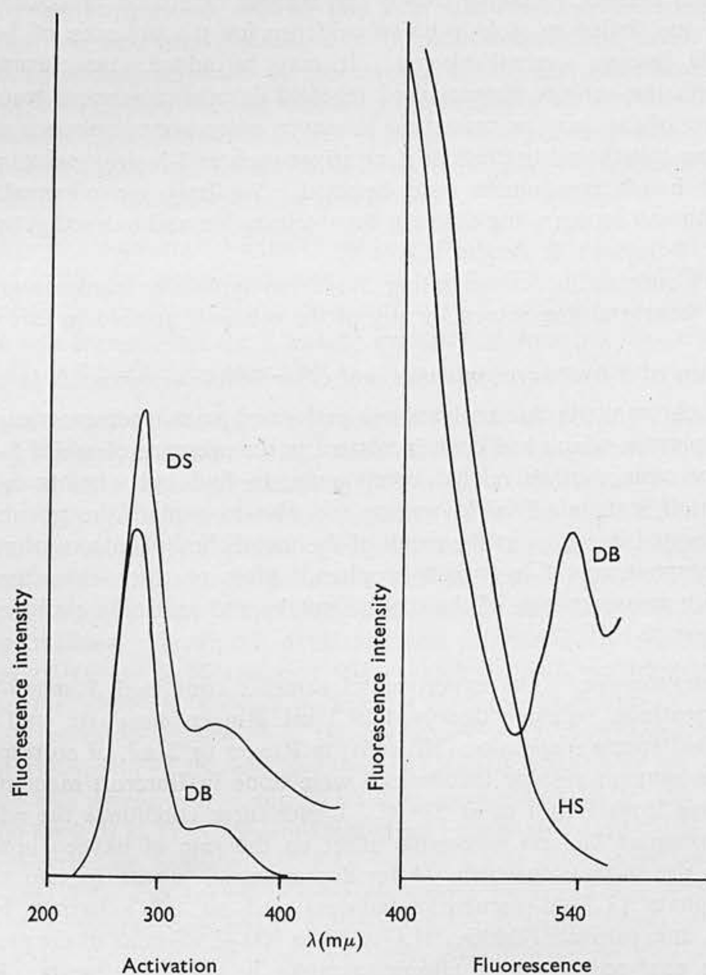


Fig. 2. Activation and fluorescence spectra of extracts from dog semen (DS), human semen (HS) and the nucleus caudatus of the dog brain (DB). The amplification used in recording the fluorescence scan of the extract from human semen (HS) was reduced to enable the record to cover the same wavelength range as that obtained with the nucleus caudatus extract (DB). If HS had contained 1 μg 5-hydroxytryptamine, a peak of the same size as that shown by DB would have been observed.

*Chromatographic observations on fresh semen*

Several attempts were made to detect 5-hydroxytryptamine by chromatography in concentrated extracts from either whole semen or freshly prepared washed sperm suspensions and seminal plasma of man, bull, ram, boar and dog. The chromatograms were examined by the various methods already cited, but no evidence was obtained for the occurrence of 5-hydroxytryptamine.

In order to repeat the observation of Katsh on the presence of 5-hydroxytryptamine in human semen, two separate experiments were carried out, with 3 ml. and 12 ml. human seminal plasma, following closely the acetone extraction procedure. These experiments, too, failed to yield positive evidence for the presence of 5-hydroxytryptamine in human seminal plasma. It may be added that chromatograms developed with the various systems used revealed several substances reacting with one or another of the spraying reagents. However, no indole compounds other than tryptophan, no substituted tryptamine derivatives such as 5-hydroxytryptamine, nor any phenolic indole compounds were detected. Similarly, we obtained negative results with human semen using different deproteinization and extraction procedures (Udenfriend, Weissbach & Brodie, 1958).

We did not succeed in demonstrating 5-hydroxytryptamine chromatographically in bull, ram, boar and dog semen by any of the methods applied in this work.

*Metabolism of 5-hydroxytryptamine and other indole compounds in semen*

A series of chromatographic analyses was performed on ram semen, washed sperm and seminal plasma, which had been incubated in the presence of added 5-hydroxytryptamine or certain other related compounds, to find out whether 5-hydroxytryptamine itself is metabolized in semen, and also to explore the possibility that it might be formed in semen as the result of the metabolic breakdown of substances such as tryptophan and 5-hydroxytryptophan. Most of these experiments were combined with measurements of the oxygen uptake and ammonia evolution during the incubations.

*5-Hydroxytryptamine.* The experimental samples contained 5 mg 5-hydroxytryptamine creatinine sulphate dissolved in 1 ml. Ringer-phosphate, and 2 ml. of either a washed sperm suspension ($10^9$ cells) in Ringer or 2 ml. of correspondingly diluted whole semen ; aerobic incubations were done in Barcroft manometers for periods ranging from 1 to 4 hr at 37° C. Under these conditions the addition of 5-hydroxytryptamine had no detectable effect on the rate of oxygen uptake, and there was no rise in free ammonia. After deproteinization with barium hydroxide and zinc sulphate {1.5 ml. incubation mixture + 0.5 ml. 0.3 N barium hydroxide + 0.5 ml. 5% zinc sulphate ($ZnSO_4.7H_2O$)}, 50 to 100 $\mu$l. aliquots of the protein-free filtrates were analysed by paper chromatography in various solvents. However, as a result of the aerobic incubation there was no evidence that either washed spermatozoa or whole semen produced any change in the 5-hydroxytryptamine content. In particular, there was no formation of 5-hydroxyindole acetic acid as an oxidative breakdown product of 5-hydroxytryptamine.

*Tryptophan.* The oxidative deamination of tryptophan by bull spermatozoa has been the subject of a study by Tosic & Walton (1950) and Tosic (1951), who have

pointed out that the formation of hydrogen peroxide during the oxidative deamination of aromatic amino-acids is responsible for the gradual decline in the oxygen uptake of incubated sperm mixtures. Using the same conditions as in the experiments with 5-hydroxytryptamine, we found that whole ram semen and washed ram spermatozoa can deaminate dl-tryptophan aerobically; moreover, whilst the same amount of ammonia was produced in the presence as in the absence of pure catalase, the rate of oxygen consumption was much higher with catalase present, owing to the decomposition of the toxic hydrogen peroxide. Chromatographic analyses of the incubation mixtures of dl-tryptophan and either whole semen or washed sperm suspensions showed that ram spermatozoa metabolize tryptophan aerobically, but not anaerobically, to several products. The major product was identified chromatographically as indoleacetic acid. The identification rested on the use of both descending and ascending chromatography in butanol + acetic acid ($R_F$ 0.88 and 0.92, respectively), as well as in two other solvents, namely, isopropanol + ammonia ($R_F$ 0.28) and potassium chloride ($R_F$ 0.60). No tryptamine, 5-hydroxytryptophan or 5-hydroxytryptamine was detected.

*5-Hydroxytryptophan.* Mixtures of sperm and 5-hydroxytryptophan were incubated under the same conditions as in experiments with 5-hydroxytryptamine and tryptophan, in the presence and absence of catalase. A small amount of ammonia was formed, but there was no evidence of decarboxylation of 5-hydroxytryptophan to 5-hydroxytryptamine.

*Tryptamine and tyramine.* No ammonia formation occurred when a solution containing 5 mg tryptamine was incubated aerobically for 3 hr at 37° C, with either a suspension of washed spermatozoa ($10^9$ cells/2 ml. Ringer) or a sample of correspondingly diluted whole ram semen. When the experiment was repeated using tyramine instead of tryptamine, only a trace of ammonia was produced. These experiments provided a good indication that the monoamine oxidase activity of ram semen must be low. Supporting evidence for this view was obtained in the following manner. Tyramine oxidation was examined by the more sensitive procedure of Green & Haughton (1961), which depends on trapping with semicarbazide the aldehyde formed during the enzymic oxidation of tyramine, and converting the semicarbazone into the corresponding 2:4 dinitrophenylhydrazone. The light absorption of the orange-coloured alkaline solution of the dinitrophenylhydrazone was determined in the Unicam spectrophotometer at 450 m$\mu$. Aerobic incubation for 2 hr at 37° C of a mixture (4 ml.) containing 1 ml. whole semen, 1 ml. 0.5 M semicarbazide solution (*p*H 7.4), 0.4 ml. 0.1 M solution of tyramine hydrochloride (*p*H 7.4), and 1.6 ml. Ringer phosphate produced less than 1/25 of the amount of semicarbazone which had been formed, under identical experimental conditions, by a preparation of mitochondria from 1 g of rat liver.

## DISCUSSION

It was thought at one time that spermatozoa deposited at ejaculation in the vagina or cervix rely on their own motility in order to traverse the uterus and reach the oviducts. More recently, however, when measurements were made of the time interval required for some spermatozoa, at any rate, to arrive at the site of fertili-

zation, this proved to be remarkably short, in some species no more than a few minutes. This and subsequent work has shown that ejaculated spermatozoa are propelled to their final destination in the oviducts not so much by their own movements as by the concomitant uterine contractions.

The mechanism responsible for the increase in uterine motility following copulation and sexual stimulation is still under investigation. Some investigators attribute it mainly to the release of oxytocin by the pituitary gland ; others believe that semen itself may provide, at least partly, the required stimulus in the form of pharmacologically active constituents. Similarly, the chemical identity of the various " oxytocic " substances in semen is still under dispute, as shown by the perusal of recent literature (Mann, 1954 ; Eliasson, 1959 ; Hawker *et al.*, 1960).

The possibility that 5-hydroxytryptamine is one of these substances has been examined. The present study does not support the claim that mammalian semen has a high content of 5-hydroxytryptamine ; our spectrophotofluorimetric as well as chromatographic analyses have shown that the amine is either altogether absent from the semen of man, bull, boar, ram and dog, or present only in minute quantities. 5-Hydroxytryptamine may, perhaps, appear in human semen in measurable quantities in special circumstances. One need only recall the well-known effect which 5-hydroxytryptamine contained in certain foods exerts on the excretion of indole derivatives in the human urine. Certain other substances administered orally can pass unchanged into the seminal plasma ; for instance, when ergothioneine, a normal constituent of the seminal plasma, is fed to a boar the administered compound is found unchanged in the ejaculated semen (Heath, Rimington, Glover, Mann & Leone, 1953). The fact remains that 5-hydroxytryptamine is not a normal constituent of seminal plasma, either in man or in ram, bull, boar and dog. It is therefore unlikely that the uterine-stimulating property of semen depends upon the presence therein of 5-hydroxytryptamine.

An indole compound which is a normal constituent of mammalian seminal plasma is tryptophan. This amino-acid, however, and the closely related 5-hydroxytryptophan, do not appear to undergo any appreciable decarboxylation in semen. 5-Hydroxytryptamine itself, and related amines such as tryptamine and tyramine, when added to semen, are also relatively stable, as shown by incubation experiments carried out with whole ejaculated semen or washed sperm suspensions.

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# DRUG-INDUCED CHANGES IN THE CONCENTRATION OF 5-OR INDOLYL COMPOUNDS IN CEREBROSPINAL FLUID AND CAUDATE NUCLEUS

BY

G. W. ASHCROFT and D. F. SHARMAN

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# DRUG-INDUCED CHANGES IN THE CONCENTRATION OF 5-OR INDOLYL COMPOUNDS IN CEREBROSPINAL FLUID AND CAUDATE NUCLEUS

BY

G. W. ASHCROFT* AND D. F. SHARMAN

*From the Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridgeshire*

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The effects of reserpine and imipramine on the concentration of indolyl compounds bearing an OR group in position 5 (5-OR indolyl compounds) in the cerebrospinal fluid and the caudate nucleus of the dog have been studied. Reserpine was shown to produce an increase in the concentration of these compounds in the cerebrospinal fluid. In the caudate nucleus reserpine produced a fall in the concentration of the basic 5-OR indolyl compounds accompanied by an equivalent increase in the concentration of acidic 5-OR indolyl compounds. No significant change in the concentration of 5-OR indolyl compounds in the cerebrospinal fluid was observed after treatment with imipramine.

In a previous publication (Ashcroft & Sharman, 1960) it was reported that there was a difference in the concentration of 5-hydroxyindolyl compounds in the cerebrospinal fluid of patients suffering from depressive psychoses and patients with neurological diseases. The present investigation on dogs was carried out to test the possibility that changes in the metabolism of 5-hydroxytryptamine in the body might be reflected in changes in the cerebrospinal fluid level of indolyl compounds bearing an OR group in position 5. Sharman (1960) has shown that many indolyl compounds bearing an OR group in position 5, where R is a hydrogen atom, or an aryl or alkyl group, and unsubstituted in the 2 position of the indole nucleus, show the fluorescence in 3N hydrochloric acid attributed to 5-hydroxyindolyl compounds by Udenfriend, Bogdanski & Weissbach (1955) and demonstrated for *N*-acetyl-5-methoxytryptamine by Axelrod & Weissbach (1961). It is possible that traces of indolyl compounds bearing an OR group in position 5 other than 5-hydroxytryptamine or 5-hydroxyindol-3-ylacetic acid are present in the cerebrospinal fluid. Because of this it was decided to refer to the substances estimated in this investigation as 5-OR indolyl compounds.

*** Present address: Department of Pharmacology, University of Edinburgh.**

## METHODS

All-glass apparatus was used for the chemical procedures. It was found that the use of rubber stoppers led to interference with the fluorescence estimations. De-ionized water was used throughout. Samples of cerebrospinal fluid (approximately 2.5 ml.) were collected into glass-stoppered tubes, each containing 5 mg ascorbic acid to prevent oxidation.

### *Estimation of the total 5-OR indolyl compounds in cerebrospinal fluid*

A measured portion (2.0 to 2.5 ml.) of cerebrospinal fluid was placed in a glass-stoppered, graduated test-tube. To the sample was added 0.5 ml. of a 10% (w/v) solution of zinc sulphate (Analar grade, recrystallized from water and dried) and the contents of the tube mixed by inversion. After the addition of 0.1 ml. of a 10% (w/v) solution of sodium hydroxide the contents of the tube were again mixed by inversion. To 2.0 ml. of the clear supernatant fluid, obtained after centrifuging for 10 min at 2,500 r.p.m., was added 1.0 ml. of concentrated hydrochloric acid containing 0.05% (w/v) ascorbic acid. The fluorescence of this solution at a wavelength of 550 mμ, when activated with ultra-violet light of wavelength 295 mμ, was measured in an Aminco-Bowman spectrophotofluorometer.

### *Estimation of 5-OR indolyl compounds in brain tissue*

*(a) Total 5-OR indolyl compounds.* The weighed tissue was homogenized in 0.1 N hydrochloric acid (2 ml./g tissue). The homogenate was then diluted to 10 ml./g tissue with water. A 5 ml. portion of the diluted homogenate was deproteinized using 1.0 ml. of a 10% solution of zinc sulphate and 0.2 ml. of a 10% solution of sodium hydroxide in a manner similar to that described for cerebrospinal fluid ; 1.0 ml. of concentrated hydrochloric acid containing ascorbic acid was added to 2.0 ml. of the clear supernatant obtained after centrifuging at 2,500 r.p.m. for 10 min. The fluorescence of this solution (activation wavelength 295 mμ ; fluorescence wavelength 550 mμ) was measured.

*(b) Basic 5-OR indolyl compounds.* A portion of the supernatant fluid obtained after the protein precipitation was extracted into butanol and estimated fluorimetrically by the method described for 5-hydroxytryptamine by Bogdanski, Pletscher, Brodie & Udenfriend (1956).

*(c) Acidic 5-OR indolyl compounds.* These were estimated by a modification of the method described for 5-hydroxyindol-3-ylacetic acid by Udenfriend, Titus & Weissbach (1955). A 2.0 ml. portion of the clear supernatant fluid obtained after protein precipitation was adjusted to a *p*H of between 1 and 2 (indicator paper) with concentrated hydrochloric acid. The solution was saturated with sodium chloride and then shaken for 1 min with 10 ml. of diethyl ether, which had been stored previously over a saturated aqueous solution of sodium sulphite.

The two phases were separated completely by centrifuging for 30 sec and the ether layer was transferred to a tube containing 1.0 ml. of 0.5 M sodium phosphate buffer *p*H 7.0. The mixture was shaken for 1 min and then the two phases were allowed to separate. A measured portion (0.8 ml.) of the aqueous layer was diluted to 1.0 ml. with water and 0.5 ml. of concentrated hydrochloric acid containing ascorbic acid was added. The fluorescence of this solution (activation wavelength 295 mμ, fluorescence wavelength 550 mμ) was measured. The fluorescence in the estimation of total, basic and acidic 5-OR indolyl compounds was measured against and expressed as ng of 5-hydroxyindol-3-ylacetic acid.

### *Collection of the cerebrospinal fluid*

Three procedures were employed for the collection of cerebrospinal fluid.

(1) Adult dogs were anaesthetized with ether followed by intravenous chloralose (70 mg/kg body weight). The head of the animal was securely fixed to allow the percutaneous introduction of a 19-gauge hypodermic needle into the cisterna magna. The needle was then secured in a rigid clamp. A narrow-bore manometer, into which some of the cerebrospinal fluid was allowed to escape, was connected to the needle via a three-way tap and gave a record of the pressure. The pressure, in mm cerebrospinal fluid, was read every 15 min. A first 2.0 to

2.5 ml. sample of cerebrospinal fluid was collected by allowing the fluid to drip from the tap into the collection tube ; further samples were only taken when the cerebrospinal fluid pressure had recovered from the fall due to sampling and had reached about 70 mm cerebrospinal fluid. This was usually a little below the initial cerebrospinal fluid pressure. In some cases the cerebrospinal fluid pressure was slow in recovering to this value, and it was found that the intravenous injection of 0.9% w/v saline accelerated recovery.

(2) A 19-gauge hypodermic needle was introduced into the cisterna magna of an anaesthetized dog as described in the preceding section.

Two ml. of cerebrospinal fluid was allowed to drip from the needle. Drugs were then injected intravenously and 2 hr later as much cerebrospinal fluid as possible was drained off in consecutive 2.0 ml. samples.

(3) A third procedure was adopted in order to examine the chronic effect of imipramine hydrochloride on the concentration of 5-OR indolyl compounds in the cerebrospinal fluid and to study the day-to-day variation in normal dogs.

Adult dogs were anaesthetized by an intravenous injection of thiopentone. A 19-gauge hypodermic needle was introduced percutaneously into the cisterna magna and 2 ml. of cerebrospinal fluid was withdrawn into a syringe. It was possible to repeat this procedure once daily, taking aseptic precautions throughout.

### *Collection of brain tissue*

At the end of each experiment the dog was bled out and the brain removed. The caudate nuclei were dissected out, frozen and stored at −17° C until analysed. The caudate nucleus was selected because this tissue has a high content of 5-OR indolyl compounds and the dissection is easily reproducible.

## RESULTS

The estimates of total 5-OR indolyl compounds in the cerebrospinal fluid of normal dogs and the effect produced by an intravenous injection of reserpine (2 mg/kg body weight) are shown in Table 1. They were obtained using the first method of cerebrospinal fluid collection. The values indicate that the increase in the cerebrospinal fluid concentration of 5-OR indolyl compounds after an intravenous injection of reserpine was greater than the increase observed to occur in successive samples taken from control animals. There was, however, no common time-course to these experiments. The time intervals between the collection of samples varied between 30 and 120 min. The second method of cerebrospinal fluid collection was introduced to standardize the timing in each experiment and to attempt to show more clearly whether there was an increase of 5-OR indolyl compounds in the cerebro-

TABLE 1

EFFECT OF RESERPINE ON THE CONCENTRATION OF TOTAL 5-OR INDOLYL COMPOUNDS IN THE CEREBROSPINAL FLUID (a)

(Cerebrospinal fluid collected by method 1)

Concentrations expressed as ng 5-hydroxyindol-3-ylacetic acid/ml. cerebrospinal fluid

| | | Concentration of total 5-OR indolyl compounds in cerebrospinal fluid | | | | | |
|---|---|---|---|---|---|---|---|
| Cerebrospinal fluid sample no.: | | 1 | 2 | 3 | 4 | 5 | 6 |
| Control dogs | 1 | 46 | 92 | 85 | 94 | 83 | — |
| | 2 | 59 | 71 | 83 | 96 | 94 | 92 |
| | 3 | 36 | 38 | 74 | 125 | 102 | — |
| Reserpine (2 mg/kg) injected intravenously after sample 2 | 1 | 49 | 87 | 72 | 108 | 168 | 161 |
| | 2 | 33 | 71 | 135 | 197 | — | — |

TABLE 2

EFFECT OF RESERPINE ON THE CONCENTRATION OF TOTAL 5-OR INDOLYL COMPOUNDS IN THE CEREBROSPINAL FLUID (b)

(Cerebrospinal fluid collected by method 2)

Concentrations expressed as ng 5-hydroxyindol-3-ylacetic acid/ml. cerebrospinal fluid

| | Concentration of total 5-OR indolyl compounds in cerebrospinal fluid | | | | Concentration of total 5-OR indolyl compounds in caudate nucleus (ng/g) |
|---|---|---|---|---|---|
| | Initial conc. | Conc. in consecutive samples taken 2 hr after initial sample | | | |
| | | 1 | 2 | 3 | |
| Control dogs | 66 | 61 | 43 | 55 | 775 |
| | 45 | 48 | 49 | 55 | 705 |
| Reserpine | 34 | 70 | 102 | 127 | 810 |
| (2 mg/kg) intravenously | 66 | 72 | 88 | 145 | 725 |
| after initial sample | 54 | 101 | 125 | 147 | 805 |

spinal fluid after reserpine. The results obtained with this procedure are shown in Table 2. They show that 2 hr after a dose of reserpine the concentration of 5-OR indolyl compounds in the cerebrospinal fluid of 3 dogs had risen to approximately twice the concentration observed in control animals. It is also shown that the level of the 5-OR indolyl compounds in the caudate nucleus had not fallen 2 hr after the injection of reserpine. In another experiment in which reserpine had been administered to two dogs over a period of 4 days (0.5 mg/kg body weight on day 1 and 0.25 mg/kg body weight on days 3 and 4), it was found that on the fifth day the concentration of 5-OR indolyl compounds in the caudate nuclei (Table 3) had fallen

TABLE 3

EFFECT OF CHRONIC ADMINISTRATION OF RESERPINE ON THE CONCENTRATION OF TOTAL 5-OR INDOLYL COMPOUNDS IN THE CEREBROSPINAL FLUID AND THE CAUDATE NUCLEUS

Concentrations expressed as ng 5-hydroxyindol-3-ylacetic acid/ml. cerebrospinal fluid or ng 5-hydroxyindol-3-ylacetic acid/g tissue

| | Concentration of total 5-OR indolyl compounds on 5th day after initial reserpine administration | | | | |
|---|---|---|---|---|---|
| Reserpine administered over 4 days | Cerebrospinal fluid samples | | | | Caudate nucleus |
| | 1 | 2 | 3 | 4 | |
| Dog 1 | 46 | 84 | 105 | — | 282 |
| Dog 2 | 98 | 83 | 83 | 89 | 262 |
| Control dog | 39 | 63 | 51 | 80 | 1,020 |

to less than half of the control values shown in Tables 2 and 3. This experiment also gave information on the concentration of 5-OR indolyl compounds in the cerebrospinal fluid after chronic reserpine administration. Consecutive 2 ml. samples of cerebrospinal fluid were obtained from each animal under ether anaesthesia. The results are shown in Table 3, and show that after the chronic administration of reserpine the concentration of 5-OR indolyl compounds in the cerebrospinal fluid is at a normal or slightly elevated level despite the greatly reduced concentration in the tissues.

The absence of a fall in the concentration of 5-OR indolyl compounds in the caudate nucleus 2 hr after an injection of reserpine suggested further investigation.

It has been shown (Paasonen & Vogt, 1956) that there is a fall in the concentration of 5-hydroxytryptamine in the caudate nucleus of the dog 4.5 hr after an injection of reserpine in a dose of 0.5 mg/kg. Brodie (1958) demonstrated that in the rabbit a dose of 1 mg/kg of reserpine will deplete 75% of the 5-hydroxytryptamine in the brain in 30 min. Recently, Brodie, Finger, Orlans, Quinn & Sulser (1960) have reported that stress will inhibit the release of 5-hydroxytryptamine from the brain after the administration of reserpine. The possibility that the stress of anaesthesia and subsequent operations was preventing the release of 5-hydroxytryptamine in the foregoing experiments was therefore investigated.

Two dogs were trained for seven days to lie quietly while the preparations for an intravenous injection were carried out. On the eighth day they were injected intravenously with reserpine (2 mg/kg body weight). Within 30 min the animals were exhibiting effects of the reserpine such as tremor, vomiting and in one case circling movements. These symptoms disappeared after 15 min and the animals became sedated. 2.5 hr after the injection of reserpine the animals were anaesthetized and killed. The concentration of total 5-OR indolyl compounds in the caudate nuclei of these two animals was 740 ng/g and 755 ng/g. This result demonstrated that the concentration of 5-OR indolyl compounds in the caudate nucleus of the dog

TABLE 4

CONCENTRATION OF 5-OR INDOLYL COMPOUNDS IN THE CAUDATE NUCLEUS OF THE DOG

Concentrations expressed as ng 5-hydroxyindol-3-ylacetic acid/g tissue

| | Total 5-OR indolyl compds | Acidic 5-OR indolyl compds | Basic 5-OR indolyl compds |
|---|---|---|---|
| Control dogs | 610 | 184 | 485 |
| | 845 | 285 | 631 |
| 2 hr after reserpine | 650 | 478 | 172 |
| (2 mg/kg intravenously) | 670 | 557 | 189 |

does not fall 2.5 hr after the injection of reserpine despite the fact that the reserpine had an obvious effect on the behaviour of the animal, and that any " stress " was avoided.

Sharman (1960) has shown that a substance behaving like 5-hydroxyindol-3-ylacetic acid can be extracted from dog brain tissue, and a second explanation was that 5-hydroxyindol-3-ylacetic acid, formed by the action of monoamine oxidase on the 5-hydroxytryptamine released by reserpine, was remaining in the brain tissue.

The following experiment was carried out on four litter-mate puppies. Two of the dogs were injected intravenously with reserpine (2 mg/kg body weight). After 2 hr the animals were anaesthetized, bled out and the caudate nuclei removed and analysed for total, acidic, and basic 5-OR indolyl compounds. The caudate nuclei from the two control dogs were analysed in a similar way. The results obtained are shown in Table 4. They show that an acidic indolyl compound, presumably 5-hydroxyindol-3-ylacetic acid, is increased by about as much as the 5-hydroxytryptamine is reduced after reserpine; the formation of this metabolite and its persistence in the tissue explain the absence of a reduction in the total 5-OR indolyl compounds 2 hr after reserpine.

*Effect of imipramine on the concentration of 5-OR indolyl compounds in the cerebrospinal fluid*

Marshall, Stirling, Tait & Todrick (1960) have demonstrated that imipramine produced a fall in the concentration of 5-hydroxytryptamine in the blood platelets in man. A reduction to about 50% of control levels was observed after this drug had been administered daily for 5 days. Costa (1960) has observed that imipramine will increase the 5-hydroxytryptamine content of several organs in the rat within 5 hr after the administration of the drug.

The effect of imipramine on the level of 5-OR indolyl compounds in the cerebrospinal fluid was examined. Preliminary experiments in which cerebrospinal fluid was collected by method 2 did not indicate acute changes in the level of 5-OR indolyl compounds in the cerebrospinal fluid after a single dose of imipramine.

The chronic effect of this drug on concentration of 5-OR indolyl compounds in cerebrospinal fluid was investigated using the third method of collection in which daily samples of cerebrospinal fluid were obtained from anaesthetized dogs. Control samples of cerebrospinal fluid were collected on four successive days from three dogs and on two successive days from one dog. After an interval of three days

TABLE 5

EFFECT OF IMIPRAMINE ON THE CONCENTRATION OF TOTAL 5-OR INDOLYL COMPOUNDS IN THE CEREBROSPINAL FLUID

Concentrations expressed as 5-hydroxyindol-3-ylacetic acid

| Dog no. | Day of expt. | Conc. of total 5-OR indolyl compounds: In cerebrospinal fluid (ng/ml. cerebrospinal fluid) | Conc. of total 5-OR indolyl compounds: In caudate nucleus (ng/g tissue) |
|---|---|---|---|
| 1 | 1 | 29 | |
| Control dog | 2 | 32 | |
| | 3 | 32 | |
| | 4 | 26 | |
| | 8 | 26 | |
| | 9 | 31 | |
| | 10 | 30 | |
| | 11 | 28 | 950 |
| 2 | 1 | 26 | |
| Control dog | 2 | — | |
| | 3 | 25 | |
| | 4 | 45 | |
| | 8 | 30 | |
| | 9 | 36 | |
| | 10 | 28 | |
| | 11 | 27 | 685 |
| 3 | 1 | 31 | |
| Imipramine | 2 | 33 | |
| started on day 5. | 6 | 49 | |
| Blood observed in | 7 | 89 | |
| cerebrospinal fluid | 8 | 118 | |
| on day 6 | 9 | 56 | 1,190 |
| 4 | 1 | 26 | |
| Imipramine started | 2 | 32 | |
| on day 7 | 3 | 26 | |
| | 4 | 25 | |
| | 8 | 30·5 | |
| | 9 | 36·0 | |
| | 10 | 35·0 | 1,065 |

two of the animals were injected intramuscularly with imipramine, twice daily, starting with 2 doses of 100 mg on the first day of injection, and increasing the doses on subsequent days by 50 mg per day to twice 250 mg on the fourth day of injection. One animal was then found to be suffering from gross oedema at the site of the injections, and was anaesthetized, bled out and the caudate nuclei dissected out from the brain. The other dog was therefore given the last dose by mouth. It was killed the following day. On each day during the experiment approximately 2 ml. of cerebrospinal fluid was obtained from each of these animals and also from the two control dogs. The results of this experiment are shown in Table 5. They show that there is only a small day-to-day variation in the concentration of 5-OR indolyl compounds in the cerebrospinal fluid of normal dogs. Bleeding into the cerebrospinal fluid occurred in one of the animals (no. 3) which was receiving imipramine. The cerebrospinal fluid on subsequent days was xanthochromic and the concentration of total 5-OR indolyl compound rose sharply probably because of the release of 5-hydroxytryptamine from blood platelets during clotting at the site of the bleeding. The observations on the remaining animal which had been treated with imipramine do not indicate any change in the concentration of total 5-OR indolyl compounds in the cerebrospinal fluid as a result of the administration of this drug. Imipramine did not produce a definite change in the concentration of total 5-OR indolyl compounds in the caudate nuclei in this experiment.

## DISCUSSION

The experiments described here have shown that changes in the concentration of total 5-OR indolyl compounds in the cerebrospinal fluid can reflect changes in the metabolism of 5-hydroxytryptamine such as those occurring after the administration of reserpine. The changes in 5-hydroxytryptamine metabolism which were presumed to occur after imipramine were not reflected by any change in the cerebrospinal fluid level of 5-OR indolyl compounds.

The change in metabolism of 5-hydroxytryptamine after a single injection of a large dose of reserpine is very great and it is possible that smaller changes might not be obvious when the acute experimental methods described here are employed. The demonstration that the release of the metabolites of 5-hydroxytryptamine is a slow process led to the conclusion that the chronic experiment would be more useful in the detection of small changes in the metabolism of 5-hydroxytryptamine, provided that the change persisted for some time. Relatively small chronic changes should be detectable because the day-to-day variation in the concentration of total 5-OR indolyl compounds in the cerebrospinal fluid of normal animals is small. It is not possible to determine from these experiments the origin of the 5-OR indolyl compound or compounds appearing in the cerebrospinal fluid after reserpine treatment.

The observations on the total, acidic and basic 5-OR indolyl compounds in the caudate nucleus show that a better evaluation of the metabolism of these compounds in the tissues can be obtained by estimating all fractions. The main acidic 5-OR indolyl compound in brain is 5-hydroxyindol-3-ylacetic acid (Sharman, 1960) and the main basic compound is 5-hydroxytryptamine (Bogdanski *et al.*, 1956). There is the possibility that other acidic and basic compounds are present in the

brain. 5-Methoxyindol-3-ylacetic acid has been isolated from the pineal gland by Lerner, Case, Biemann, Heinzelman, Szmuszkovicz, Anthony & Krivis (1959), and other 5-OR indolyl derivatives such as *N*-acetyl-5-methoxytryptamine have also been isolated (Lerner, Case & Heinzelman, 1959). Because the methods used here would not distinguish all of these compounds it was found preferable to describe the substances by the general term of 5-OR indolyl compounds.

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# THE EFFECT OF α-METHYLDOPA ON THE METABOLISM OF 5-HYDROXYTRYPTAMINE IN RAT BRAIN

D. F. SHARMAN and S. E. SMITH
Pharmacology Unit, A.R.C. Institute of Animal Physiology, Babraham, Cambridge, and Department of Pharmacology and Therapeutics, St. Thomas's Hospital Medical School, London, S.E.1

(*Received* 21 *March* 1962)

ADMINISTRATION to mice and guinea pigs of α-methyl-dihydroxyphenylalanine (α-methyldopa), an inhibitor of dihydroxyphenylalanine (dopa) decarboxylase and 5-hydroxytryptophan decarboxylase, has been shown to reduce the concentration of 5-hydroxytryptamine (5-HT) in the brain (SMITH, 1960). This has been confirmed by other workers using these and other species, and it has since been found that depletion of the noradrenaline in the brain also occurs (KUNTZMAN, COSTA, GESSA, HIRSCH and BRODIE, 1961). There is a difference in the behaviour of the 5-HT and the noradrenaline levels after administration of α-methyldopa; whereas 5-HT concentrations remain low for a few hours only, noradrenaline levels remain low for several days (HESS, OZAKI and UDENFRIEND, 1960). These findings indicate that 5-HT and noradrenaline are either depleted by different mechanisms, or that there is a difference in the rate of restoration of the stores of the two amines; or both may be true. It seems likely that 5-HT depletion results from inhibition of 5-hydroxytryptophan decarboxylase, because the time course of the depletion coincides roughly with the presence of the α-methyldopa in the brain (MURPHY and SOURKES, 1959). There is no such correlation in the case of noradrenaline depletion and it has been suggested that noradrenaline is released from its binding sites in the brain by α-methyldopa or by its metabolites (CARLSSON, 1961).

The following investigations were made to find out whether evidence could be obtained of the release of stored 5-HT after administration of α-methyldopa. Evidence that release of 5-HT in the body can be detected by an increase in the concentration of 5-hydroxyindolylacetic acid in the urine has been presented by SHORE, SILVER and BRODIE (1955). ASHCROFT and SHARMAN (1961) have shown that there is also an increase in the acidic 5-OR indolyl compounds (where R is hydrogen, an alkyl or an aryl group), presumably of 5-hydroxyindolylacetic acid, in the brain of the dog, after an injection of reserpine. The possibility that α-methyldopa might produce a similar increase in the acidic 5-OR indolyl compounds in the brain was therefore studied.

## METHODS

Groups of female albino rats were given intraperitoneal injections of saline or α-methyldopa (400 mg/kg) and killed at intervals thereafter. The cerebellum and a large portion of the cerebral cortex were removed from the brain and an extract of the remaining tissue was prepared by deproteinising a dilute acid homogenate with zinc sulphate and sodium hydroxide. A portion of this solution was made 3 N with respect to hydrochloric acid and the fluorescence was measured in an Aminco-Bowman spectrophotofluorometer. (Activation wavelength 285 mμ; fluorescence wavelength 540 mμ. Uncorrected instrumental values.) This gives an estimate of the concentration of total 5-OR indolyl compounds present in the tissue. The basic 5-OR indolyl compounds were extracted from a portion of the deproteinised solution by the method described for 5-HT (BOGDANSKI, PLETSCHER,

BRODIE and UDENFRIEND, 1956). The extraction of acidic 5-OR indolyl compounds was carried out by the method described for 5-hydroxyindolylacetic acid (UDENFRIEND, TITUS and WEISSBACH, 1955). The fluorimetric estimation of both the acidic and basic fractions was similar to that described for the total 5-OR indolyl compounds.

## RESULTS AND DISCUSSION

Any difference between the measured total and the total obtained by summation of the two fractions would represent neutral and amphoteric 5-OR indolyl compounds present in the tissue. This would include 5-hydroxytryptophan.

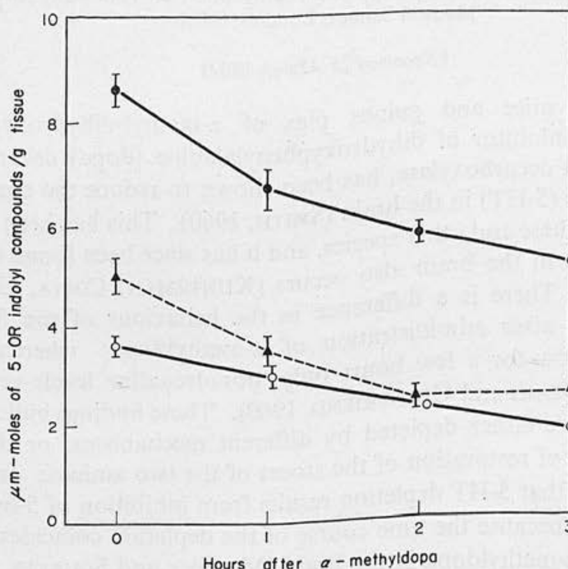


FIG. 1.—Rat brain 5-OR indolyl compounds after administration of α-methyldopa (400 mg/kg) at zero time. ●—● Total 5-OR indoles. ▲---▲ Basic fraction. ○—○ Acidic fraction. Points are means of 8 results. Vertical lines represent S.E.M. Absence of a vertical line indicates that the standard error is smaller than the symbol used.

The results were calculated as μm-moles of 5-OR indolyl compounds per g brain. It can be seen (Fig. 1) that the concentrations of total, basic and acidic 5-OR indolyl compounds decreased progressively with time after injection of α-methyldopa.

There is no evidence of the release of 5-HT because, in spite of a large decrease in the basic fraction, the concentration of the acidic fraction did not increase as it did after reserpine (ASHCROFT and SHARMAN, 1961). The acidic fraction diminished continuously suggesting that the utilization of the stored 5-HT was falling continuously. There was a significant difference between the measured total and the sum total of the basic and acidic fractions at 2 hr ($P < 0{\cdot}05$) and 3 hr ($P < 0{\cdot}01$) after treatment. This indicates accumulation of a very small amount of a neutral or amphoteric 5-OR indolyl compound, possibly 5-hydroxytryptophan. COOPER and MELCER (1961) suggest that the brain derives its supply of 5-hydroxytryptophan from the circulation. If this uptake were inhibited by α-methyldopa, this would lead to a diminished amount of 5-HT in the tissue, but not to an accumulation of 5-hydroxytryptophan such as one might expect to occur if inhibition of dopa decarboxylase were the sole action of the drug.

Further interference by α-methyldopa with the metabolism of the 5-OR indolyl series of compounds could occur if α-methyldopa or its metabolites interfered with monoamine oxidase. The possible inhibitory actions of α-methyldopa, α-methyl-*m*-tyrosine (another decarboxylase inhibitor), α-methyldopamine, α-methyl-*m*-tyramine (their decarboxylated derivatives), Cobefrine and metaraminol (the β-hydroxylated amine derivatives) on rat brain monoamine oxidase were investigated manometrically using 5-HT as substrate. Results of these experiments (Table 1) show that the amino acids themselves had no inhibitory effect, the β-hydroxylated amines had very little effect, but both α-methyldopamine and α-methyl-*m*-tyramine showed some inhibitory activity. These results are consistent with the predictions made by BLASCHKO (1940) for this type of compound.

Theoretically therefore, the decarboxylation metabolite could interfere with the metabolism of 5-HT by inhibiting monoamine oxidase, although the concentration required appears to be large. Any such inhibition would limit the decrease in the basic fraction and cause a decrease in the acidic 5-OR indolyl fraction. Such changes were observed between 2 hr and 3 hr after treatment with α-methyldopa, but they are not necessarily due to inhibition of monoamine oxidase.

It is apparent that α-methyldopa could influence the metabolism of 5-HT in the brain in four ways:—

1. Inhibition of the decarboxylation of 5-hydroxytryptophan.
2. Inhibition of monoamine oxidase by α-methyldopamine, a metabolite of α-methyldopa.
3. Inhibition of uptake of 5-hydroxytryptophan.
4. Displacement or release of 5-HT by α-methyldopa or its metabolites.

TABLE 1.—INHIBITION OF RAT BRAIN HOMOGENATE MONOAMINE OXIDASE BY VARIOUS COMPOUNDS*

| Inhibitor | Concentration | % Inhibition |
|---|---|---|
| α-Methyldopa | $5 \times 10^{-3}$ M | 0 |
| α-Methyl-*m*-tyrosine | $5 \times 10^{-3}$ M | 0 |
| α-Methyldopamine | $5 \times 10^{-4}$ M | 32 |
| | $5 \times 10^{-3}$ M | 76 |
| α-Methyl-*m*-tyramine | $5 \times 10^{-4}$ M | 27 |
| | $5 \times 10^{-3}$ M | 68 |
| Cobefrine | $5 \times 10^{-3}$ M | 14 |
| metaraminol | $5 \times 10^{-3}$ M | 13 |

* Manometric investigations using $5 \times 10^{-3}$ M-5-hydroxytryptamine as substrate.

Whereas mechanism 1 would be sufficient to explain the results obtained in this work, a contribution by mechanisms 2 and 3 is not ruled out. The evidence is against the liberation of 5-HT (mechanism 4) because no increase in the concentration of acidic 5-OR indolyl compounds was observed.

## SUMMARY

The effect of α-methyldopa on the metabolism of 5-HT in the brain of the rat has been studied. No evidence was obtained to suggest that this drug causes an increased release of 5-HT in the brain. Other ways in which the metabolism of 5-HT might be affected by this drug are considered.

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# THE ACTION OF 2-AMINO-TETRALIN (β-TETRAHYDRONAPHTHYLAMINE) ON THE METABOLISM OF 5-HYDROXYTRYPTAMINE IN THE BRAIN OF THE MOUSE

BY

**D. ROBINSON and D. F. SHARMAN**

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# THE ACTION OF 2-AMINO-TETRALIN (β-TETRAHYDRONAPHTHYLAMINE) ON THE METABOLISM OF 5-HYDROXYTRYPTAMINE IN THE BRAIN OF THE MOUSE

BY

D. ROBINSON AND D. F. SHARMAN

*From the Pharmacology Unit, Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge*

(*Received November* 29, 1966)

It has been shown that the administration of 2-amino-tetralin (β-tetrahydronaphthylamine) to the mouse results in a decrease in the concentration of 5-hydroxyindol-3-ylacetic acid, an acid metabolite of 5-hydroxytryptamine, in the brain (Sharman, 1966). This report attempts to explain the mechanism by which this change is brought about.

## METHODS

All chemicals and reagents were of analytical reagent quality.

The following drugs were used: L-α-methyl-3,4-dihydroxyphenylalanine (α-methyldopa), Merck, Sharp and Dohme. 2-Amino-tetralin hydrochloride (2-amino-1,2,3,4-tetrahydronaphthalene hydrochloride; β-tetrahydronaphthylamine hydrochloride), Dr. Theodor Schuchardt G.m.b.H. Pheniprazine (JB 516; β-phenylisopropylhydrazine hydrochloride), Lakeside Laboratories Inc. L-tryptophan, Hopkins & Williams Limited.

Drugs were injected intraperitoneally dissolved in 0.9% sodium chloride solution except for L-tryptophan which was triturated in 0.9% sodium chloride in an agate mortar to yield a very fine suspension of the amino acid (Ashcroft, Eccleston & Crawford, 1965).

Male albino mice, 20–35 g wt. were used throughout. The tissue used for analysis was obtained as follows: the animals were stunned, decapitated and the brains taken out and placed on a glass plate in an ice bath. Olfactory bulbs, cerebellum, medulla and pons were removed, and the remaining brain tissue from three mice was combined for each estimation. The tissue was homogenised in 2 vol. of 0.1 N-hydrochloric acid together with 5–10 mg ascorbic acid in a Griffith pattern glass tissue grinder and the homogenate diluted with 1 vol. of water. One millilitre of 16% zinc sulphate ($ZnSO_4.7H_2O$) and 0.1 ml. 20% sodium hydroxide were added and the homogenate was centrifuged for 5 min at 3,500 rev/min. The supernatant fluid was filtered to remove floating fatty material and the precipitate washed with water. After centrifuging, the washings were also filtered and combined with the first supernatant fluid. The total volume was 6 ml. Of this, 4 ml. was used to estimate 5-hydroxyindol-3-ylacetic acid and 2 ml. was used to estimate 5-hydroxytryptamine. 5-Hydroxyindol-3-ylacetic acid was estimated fluorimetrically as acidic 5-OR indolyl compounds (Ashcroft & Sharman, 1962). 5-Hydroxytryptamine was estimated, in principle, as described by Bogdanski, Pletscher, Brodie & Udenfriend (1956).

The results are shown in Fig. 2. Tryptophan given in doses of 100 mg/kg and 400 mg/kg caused a significant increase in the concentration of 5-hydroxyindol-3-ylacetic acid ($P<0.01$) but only the larger dose of the amino acid produced a small but significant ($P<0.01$) increase in 5-hydroxytryptamine. The administration of 2-amino-tetralin in combination with tryptophan reduced the increase in 5-hydroxyindol-3-ylacetic acid. In addition there was now a significant increase ($P<0.01$) in 5-hydroxytryptamine above control values with both doses of tryptophan.

*The effect of pheniprazine and α-methyldopa*

The effect of the monoamine oxidase inhibitor pheniprazine (2 mg/kg and 5 mg/kg) and of α-methyldopa (400 mg/kg), alone and combined, on the concentrations of 5-hydroxyindol-3-ylacetic acid and 5-hydroxytryptamine in the brain of the mouse are

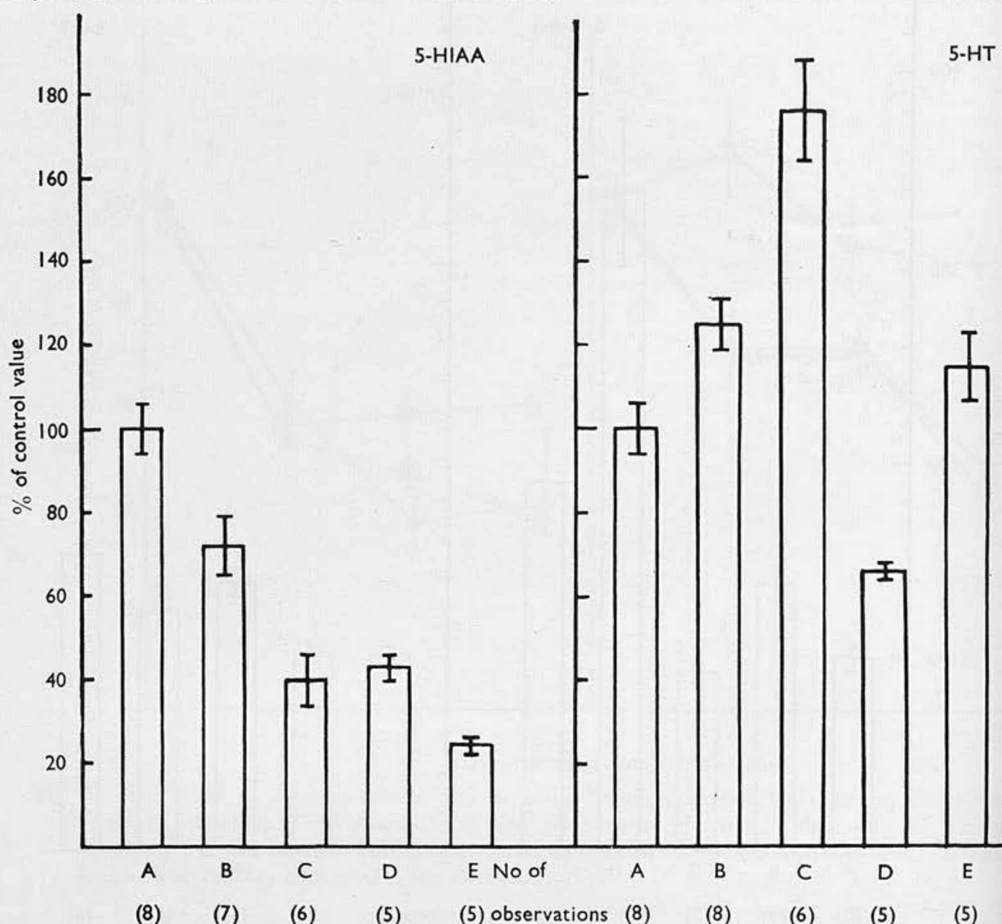


Fig. 3. The effect of pheniprazine and α-methyl-3,4-dihydroxyphenylalanine on the concentrations of 5-hydroxyindol-3-ylacetic acid and 5-hydroxytryptamine in the brain of the mouse. A=control; B=pheniprazine (2 mg/kg); C=pheniprazine (5 mg/kg); D=α-methyldopa (400 mg/kg); E=α-methyldopa (400 mg/kg) and pheniprazine (5 mg/kg). Results are given as means ± s.e.m. expressed as a percentage of the mean control value. The observations were made 2 hr after the administration of the drugs.

shown in Fig. 3. Like 2-amino-tetralin (Fig. 1) pheniprazine lowered the tissue content of 5-hydroxyindol-3-ylacetic acid, but produced a significant accumulation of 5-hydroxytryptamine, which did not occur after 2-amino-tetralin. When doses of $\alpha$-methyldopa and of pheniprazine, which alone produced equal falls in the concentration of 5-hydroxyindol-3-ylacetic acid, were combined the effects on the acid were additive (Fig. 3). The same combination of drugs also had additive effects on the tissue content of 5-hydroxytryptamine, the rise produced by pheniprazine and the fall caused by $\alpha$-methyldopa cancelling each other out. This was not observed when 2-amino-tetralin and $\alpha$-methyldopa were combined.

## DISCUSSION

5-Hydroxyindol-3-ylacetic acid is formed from 5-hydroxytryptamine by the action of the enzymes monoamine oxidase and aldehyde dehydrogenase. The simplest explanation for a decrease in this acid metabolite after 2-amino-tetralin would be inhibition of these enzymes. However, the fall in the concentration of 5-hydroxyindol-3-ylacetic acid in the brain should then be accompanied by an increase in 5-hydroxytryptamine. The administration of the known monoamine oxidase inhibitor pheniprazine caused a signicant increase in the concentration of 5-hydroxytryptamine even with a dose that produced a fall in the concentration of 5-hydroxyindol-3-ylacetic acid smaller than that seen after 2-amino-tetralin. However, no increase in the amine was seen after 2-amino-tetralin. That the effect of 2-amino-tetralin is due to inhibition of the enzyme aldehyde dehydrogenase is unlikely since it has been shown that the formation of homovanillic acid, an acid metabolite of dopamine is, in fact, accelerated in the mouse brain by the administration of 2-amino-tetralin (Sharman, 1966). The evidence thus suggests that the enzymic processes by which 5-hydroxytryptamine is oxidized to 5-hydroxyindol-3-ylacetic acid are unaffected by 2-amino-tetralin.

Ashcroft *et al.* (1965) and Eccleston, Ashcroft & Crawford (1965) have shown how tryptophan loading of animals can be used to study the intermediate metabolism of 5-hydroxyindole compounds in tissues. The present results with tryptophan loading suggest that the ability to synthesize 5-hydroxytryptamine in the brain is not arrested by 2-amino-tetralin since there is an increased level of 5-hydroxytryptamine when both tryptophan and 2-amino-tetralin are given together. The reduced formation of 5-hydroxyindol-3-ylacetic acid, however, suggests that some step in the conversion of 5-hydroxytryptamine to 5-hydroxyindol-3-ylacetic acid is impaired by the administration of 2-amino-tetralin.

A possible point in this conversion which might be affected is the rate of release and transport of the amine to a site where it can be metabolized to the acid. The rate at which an amine is being used in the brain can be studied by blocking its synthesis and measuring the rate of its disappearance from the tissue. $\alpha$-Methyldopa blocks the synthesis of 5-hydroxytryptamine in the brain (Smith, 1960). The present experiments show that the rate of fall of 5-hydroxytryptamine after $\alpha$-methyldopa is unaffected by the simultaneous administration of 2-amino-tetralin, and thus it would appear that the rate of release of 5-hydroxytryptamine from its storage sites is proceeding at a normal rate.

Yet this need not be so when 2-amino-tetralin is given alone. Assuming this compound inhibited the release of 5-hydroxytryptamine, one would not only have expected that the decrease in tissue 5-hydroxytryptamine following the administration of $\alpha$-methyldopa would be checked by 2-amino-tetralin, but also that there would have been a larger fall in tissue 5-hydroxyindol-3-ylacetic acid when the two drugs were given together.

No such additive effects would be expected, however, if the release of 5-hydroxytryptamine by 2-amino-tetralin were inhibited by a reflex response which counteracts or balances a direct effect of the drug on some other neural mechanism. Such a reflex response may not be elicited in the presence of $\alpha$-methyldopa because the release of 5-hydroxytryptamine is already sufficiently lowered by inhibition of synthesis. Thus the effect of a combination of the two drugs on the concentration of 5-hydroxytryptamine would not be additive.

The effects of 2-amino-tetralin on the metabolism of monoamines in the brain are not the same in different species of animals. Juorio & Vogt (unpublished) have shown that in the rat this drug produces changes similar to those seen in the mouse, whereas in the pigeon the levels of both dopamine and 5-hydroxytryptamine and their acid metabolites were depressed.

## SUMMARY

1. The administration of 2-amino-tetralin ($\beta$-tetrahydronaphthylamine) to the mouse causes a fall in the concentration of 5-hydroxyindol-3-ylacetic acid in the brain without changing the concentration of 5-hydroxytryptamine.

2. A comparison of the effect of 2-amino-tetralin with the fall in 5-hydroxyindol-3-ylacetic acid and the increase in 5-hydroxytryptamine produced by pheniprazine, a known inhibitor of monoamine oxidase, shows that the change in 5-hydroxyindol-3-ylacetic acid caused by 2-amino-tetralin is not a result of inhibition of this enzyme.

3. The effect of 2-amino-tetralin on the cerebral concentrations of 5-hydroxyindol-3-ylacetic acid and 5-hydroxytryptamine in mice given a loading dose of tryptophan suggested that it is a slowing rate of utilization of 5-hydroxytryptamine that plays the major role in causing the reduction of 5-hydroxyindol-3-ylacetic acid. However, when the synthesis of 5-hydroxytryptamine was inhibited by $\alpha$-methyl-3,-4-dihydroxyphenylalanine the administration of 2-amino-tetralin did not appear to reduce the rate at which 5-hydroxytryptamine disappeared from the brain.

4. It is suggested that the effect of 2-amino-tetralin on the cerebral metabolism of 5-hydroxytryptamine in the mouse might be an indirect effect of the drug. Because the inhibition of synthesis of 5-hydroxytryptamine by $\alpha$-methyl-3,4-dihydroxyphenylalanine brings about a reduction in the rate of utilization of the amine, the effects of a combination of these two drugs need not be additive.

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# CHEMICAL AND PHYSIOLOGICAL CHANGES PRODUCED BY ARTERIAL INFUSION OF DIHYDROXYPHENYLALANINE INTO ONE CEREBRAL HEMISPHERE OF THE CAT

R. DAGIRMANJIAN,* R. LAVERTY,† P. MANTEGAZZINI,
D. F. SHARMAN and M. VOGT
Agricultural Research Council Institute of Animal Physiology,
Babraham, Cambridge

(*Received* 15 *October* 1962)

MANTEGAZZINI and GLÄSSER (1960) have shown that injection of dihydroxyphenylalanine (DOPA)‡ into an external cartoid artery of a *cerveau isolé* preparation of the cat caused arousal, whereas the intravenous injection of the same amount had no such effect. The injection into the cartoid artery of dopamine, the amine formed in the body from DOPA by decarboxylation, did not produce arousal. It is known that dopamine does not readily penetrate the blood-brain barrier, whereas DOPA passes more easily. The conclusion drawn by MANTEGAZZINI and GLÄSSER was that either DOPA itself, or one of its metabolites formed in the brain, were responsible for the arousal. The present experiments were undertaken in order to try and trace the chemical changes produced in the brain by the arterial infusion of DOPA. The technique was modified in such a way that the DOPA was trapped essentially in one half of the brain; thus it became possible to detect any changes resulting from the infusion by a comparison of the chemical composition of the two sides.

## METHODS

A preliminary series of experiments was done on cats anaesthetized with pentobarbitone sodium. With this technique no information can be obtained from the electroencephalogram, since the anaesthetic causes a persistent sleep pattern, but chemical estimations can be carried out. In the main experiments the *cerveau isolé* was prepared by the method of MANTEGAZZINI and FERRARA (1960). Under ether anaesthesia a hole of about 1 $cm^2$ was drilled in the skull just anterior to the occipital protuberance. The brain was then transected by means of a spatula oriented stereotaxically so as to cut in a plane inclined at 45° and passing dorsally through the inferior colliculi and ventrally through the midbrain just anterior to the border of the pons. After this transection the EEG shows a pattern of sleep "spindles" and the pupils are maximally constricted.

The arterial infusion of DOPA was made through fine polythene tubing tied into the right lingual artery, the tip of the tubing lying at the junction of the lingual and carotid arteries. A ligature around the lingual artery fixing the tip of the catheter very near this junction ensured that none of the infused fluid escaped into branches of the lingual artery instead of entering the carotid blood stream. The tubing was filled with heparinized saline and firmly clamped to avoid blood entering it and a clot forming before the infusion was started. By infusing a 0·5% solution of DOPA at a rate of only about 0·1 ml/min any interference with the circulation to the brain was avoided. The drug was mainly distributed to the side of the infusion since the infusion was too slow to cause any local rise in pressure. Before infusing the DOPA a test injection was made of a single dose of a few μg adrenaline; pupillary dilation restricted to the side of the injection indicated that there was little or no mixing of the blood supply to the iris.

* Riker Fellow.
† Beit Memorial Fellow.
‡ Abbreviation used: DOPA, dihydroxyphenylalanine.

The electroencephalograms were obtained by inserting metal screws into two small drill holes made symmetrically in the skull above the parietal cortex and connecting these to an Ediswan EEG recorder. One or more hr were allowed to elapse between the brain transection and the first recordings. As soon as control recordings had been made, infusion of DOPA was begun and pupils and EEG observed. The experiments were normally terminated when changes had occurred on the side of the infusion; this usually required no less than 15 min. When no difference between the two sides was observed, the infusion was stopped after 45 min and the brain excised. It was cut in the midline and each side analysed separately. In one series of experiments, dopamine was estimated in the caudate nuclei, and noradrenaline in the hypothalamus. In the remaining experiments dopamine was not only estimated in the caudate nuclei but also in the tissue containing the reticular formation. When the preparation was a *cerveau isolé*, the regions examined were hypothalamus, mesencephalic central grey, and tegmentum. When the brain had not been transected, the region containing the upper part of the bulbar reticular formation was included in the pooled tissues. In these experiments noradrenaline was not estimated.

*Dopamine estimations*. The tissue was quickly weighed and homogenized in twice its weight of 0·1 N-HCl to which either a little ascorbic acid or a few crystals of potassium metabisulphite ($K_2S_2O_5$) had been added. The homogenate was diluted with water to five times its volume and the protein precipitated with an equal volume of 0·8 M-$HC10_4$. Removal of the perchloric acid and adsorption of the dopamine to a column of Dowex 50-X 8 was then carried out as described by BERTLER, CARLSSON, ROSENGREN and WALDECK (1958). The column was washed with 6–10 ml 0·4 N-HCl to remove any DOPA and noradrenaline, and the dopamine eluted with 6 or 8 ml 2N-HCl. Estimation of the dopamine was carried out fluorimetrically after condensation with ethylene diamine according to the procedure of WEIL-MALHERBE and BONE (1952, 1957). Recovery of dopamine added to a sample of the original homogenate was determined in every experiment, and correct development of fluorescence was ascertained by estimating the fluorescence produced by a known amount of dopamine added to a fraction of the final eluate. Recoveries amounted to about 70% when the higher concentrations in the caudate nucleus were estimated, and to approximately 35% from the hypothalamus and midbrain where the concentration is very near the threshold of the method: when the size of the column was increased, and a new batch of resin employed, recovery of the small amounts rose to that of the larger quantities.

*Noradrenaline estimations*. The majority of the estimations were done by bioassay on the pithed rat's blood pressure after chromatography on paper in a phenol–HCl system (VOGT, 1952, 1953, 1954; MUSCHOLL and VOGT, 1957). Some of the control experiments, however, were done by fluorimetry, and the procedure adopted was identical with that described by SHARMAN, VANOV and VOGT (1962). It consisted essentially in the adsorption to the resin Dowex 50-X 8 (BERTLER *et al.*, 1958) followed by elution and fluorimetric estimation of the trihydroxyindole derivative of noradrenaline (VON EULER and FLODING, 1955; VON EULER and LISHAJKO, 1961). In ten such experiments, in which DOPA had not been given, the hypothalamus or midbrain of one hemisphere was analysed by fluorimetry, that of the other by bioassay. In seven of these parallel estimations the concentrations found, after correction for recovery, were within 13% of each other. In the three remaining instances, the result of the chemical estimation was 42, 50, and 75% respectively above that of the biological assay. Since the aberrant figures were all obtained on one day, there was probably some uncontrolled factor operating on this particular occasion. However, attempts at using the fluorimetric estimation for experiments in which DOPA had been infused gave values which were much higher than those obtained by bioassay, and it was concluded that enough DOPA was retained in the column and eluted with the noradrenaline to render the method unsuitable.

## RESULTS

A first group of five experiments (Table 1) in which DOPA was given into the right lingual artery was carried out on cats under pentobarbitone. It gave preliminary information on the changes in catecholamines produced in the brain. The dopamine of the caudate nucleus was higher on the side of the infusion, but the rise was large in only two experiments. The concentration on the contralateral side was within the normal limits shown in three control experiments (Nos. 7–9), in which cats were either only anaesthetized or given an infusion of saline into the right lingual artery. The hypothalamic noradrenaline concentration was a little higher on the side of the DOPA infusion, but the largest increase of 36% was at the very limit of the discrimination of the method of assay. Noradrenaline concentrations in the midbrain were not significantly different on the two sides.

Table 2 summarizes the next series of experiments in which the brain had been transected and EEG records were taken. In all cats there was some mydriasis on the

TABLE 1.—CATS IN PENTOBARBITONE ANAESTHESIA

| Cat No. | DOPA infused mg | DOPA infused min | Dopamine in caudate nucleus L | Dopamine in caudate nucleus R | Dopamine in caudate nucleus % rise | Noradrenaline in hypothalamus L | Noradrenaline in hypothalamus R | Noradrenaline in hypothalamus % rise | Noradrenaline in 'midbrain'† L | Noradrenaline in 'midbrain'† R |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 15 | 30 | 11·3 | 14·3 | 27 | 1·1 | 1·5 | 36 | | |
| 2 | 20 | 42 | 8·1 | 19·7 | 145 | — | — | — | | |
| 3 | 15 | 33 | — | — | — | 1·3 | 1·6 | 23 | | |
| 4 | 15 | 30 | 11·5 | 12·9 | 12 | 1·1 | 1·5 | 36 | 0·17 | 0·16 |
| 5 | 15 | 31 | 9·5 | 15·0 | 58 | 0·9 | 1·0 | 6 | 0·08 | 0·12 |
| 7 | 0 | Exsanguinated under chloroform | 6·9 | 6·8 | | 1·5* | 1·4 | | 0·12 | 0·12* |
| 8 | 0 | Pentobarbitone anaesthesia | | 11·6 | | 1·3* | 1·2 | | | |
| 9 | 0 | Infusion of NaCl into r. lingual artery under pentobarbitone | 8·7 | 8·6 | | 1·1 | 1·1 | | 0·21 | 0·20 |

Catecholamine concentrations (μg/g fresh tissue) in different parts of the brain after an infusion of dihydroxyphenylalanine (DOPA) into the right lingual artery. The figures have not been corrected for recovery which was approx. 70% throughout.

* Chemical estimation.

† "Midbrain" = tegmentum and periaqueductual grey.

side of the infusion; the dilated pupil responded to light; this response has been interpreted by MANTEGAZZINI and FERRARA (1960) as a central inhibition of oculomotor tone due to the awakening effect of the drug. The mydriasis was followed in cats 1, 3 and 4 by electroencephalographic arousal which was either much more pronounced on, or restricted to, the side of the infusion. The chemical findings showed that the dopamine content of the right caudate nucleus was more than twice that of the left, but that the noradrenaline in the hypothalamus had not risen on the side of the infusion.

Judging by the physiological and chemical changes, it appears that, in these experiments, the effects of the infusion of DOPA were more completely restricted to the side of the infusion than in the experiments of Table 1 in which the brain was not transected.

TABLE 2.—*Cerveau isolé* PREPARATION

| Cat No. | DOPA infuse mg | DOPA infuse min | Dopamine in caudate nucleus L | Dopamine in caudate nucleus R | Dopamine in caudate nucleus % rise | Noradrenaline in hypothalamus L | Noradrenaline in hypothalamus R | Noradrenaline in hypothalamus % rise | Arousal in right hemisphere |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 5 | 6 | 5·7 | 13·1 | 130 | 1·4 | 1·5 | 7 | + |
| 2 | 15 | 19 | 7·5 | 15·7 | 109 | 1·1 | 1·1 | 0 | — |
| 3 | 20 | 22 | 6·9 | 16·4 | 138 | 1·1 | 1·1 | 0 | + |
| 4 | 20 | 30 | 6·2 | 14·8 | 138 | 1·3 | 1·3 | 0 | ++ |

Catecholamine estimations (μg/g fresh tissue) in different parts of the brain after an infusion of dihydroxyphenylalanine (DOPA) into the right lingual artery. The figures have not been corrected for recovery which was approx. 70% throughout.

It is possible that the destruction of vessels inside the midbrain reduces the opportunity for exchange of blood between the two sides.

In the caudate nucleus, dopamine is the dominant catecholamine; in this region of the brain the oxidation of dopamine to noradrenaline apparently does not occur. In the hypothalamus and midbrain, normal dopamine concentrations are very small indeed (BERTLER and ROSENGREN, 1959), and noradrenaline concentrations are high. The dopamine in these regions may be no more than a precursor of noradrenaline. It was of interest to know whether the infusion of DOPA only increased the dopamine at the sites where it represents the final product of synthesis, or whether changes might

TABLE 3.—DOPAMINE ESTIMATIONS ($\mu$g/g FRESH TISSUE) IN DIFFERENT PARTS OF THE CAT'S BRAIN AFTER AN INFUSION OF DIHYDROXYPHENYLALANINE (DOPA) INTO ONE LINGUAL ARTERY

| | | | Dopamine in | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Caudate nucleus | | | Hypothalamus and "reticular formation"* | | | |
| Cat No. | DOPA mg | infused min | control side | side of infusion | % rise | control side | side of infusion | % recovery | % change |
| 1 | 15 | 30 | 8·1 | 10·4 | 28 | 0·21 | 0·15 | 30 | −29 |
| 2 | 15 | 28 | 11·1 | 17·5 | 58 | 0·14 | 0·35 | 30 | 150 |
| 3 | 15 | 31 | 9·2 | 10·7 | 16 | 0·38 | 0·49 | 36 | 29 |
| 4 | — | — | — | — | | 0·20 | | 35 | |
| 5 | 18 | 38 | 10·9 | 12·6 | 16 | 0·15 | 0·20 | ? | 33 |
| 6 | 14 | 28 | 10·7 | 16·4 | 53 | 0·58 | 0·82 | 69 | 41 |
| 7 | 12 | 26 | 10·2 | 17·5 | 73 | 0·55 | 1·67 | 82 | 204 |

Experiments 1–3 under pentobarbitone anaesthesia, experiments 5–7 on the *cerveau isolé* preparation. Experiment 4, control figures for untreated cat. None of the figures are corrected for recovery. Recoveries from the caudate nuclei about 70%. Changes below 0·1 $\mu$g/g are not significant.

* Consisting of tegmentum, periaqueductual grey and region of upper bulbar reticular formation in experiments 1–4; of tegmentum and periaqueductual grey only, in the remaining experiments.

also occur in the parts where it may be only a precursor. Increases of dopamine in all parts of the brain had been found by BERTLER and ROSENGREN (1959) after the intravenous administration of DOPA to rabbits, but intravenous injections are not strictly comparable with arterial infusions in which much of the DOPA is immediately trapped by the tissue.

A third series of experiments was therefore carried out, in which the dopamine was not only estimated in the caudate nuclei but also in the noradrenaline-rich parts of the brain, such as hypothalamus and midbrain. As before, differences in dopamine content between left and right caudate nucleus were more pronounced in experiments carried out on the *cerveau isolé* (experiments 5–7, Table 3) than in those done on the intact brain under pentobarbitone anaesthesia. Concentrations of dopamine in the hypothalamus and midbrain reticular formation are so low that the accuracy of the estimation is less than that for the caudate nucleus. Table 3 shows that the recoveries of dopamine, added in amounts similar to those present in the mesencephalon, were at first much lower than in the tissues containing larger quantities. However, as explained under "methods," the recoveries were greatly improved in expts. 6 and 7 and were similar for all quantities of dopamine.

In all experiments, more dopamine was found in hypothalamus and midbrain on the side of the infusion than on the control side. The increase was largest when the

difference between the two caudate nuclei was most pronounced, but the highest absolute amount observed (1·6 μg/g) was still only a small fraction of the content of the normal caudate nucleus.

## DISCUSSION

The most striking chemical difference between the two sides of the brain after unilateral infusion of DOPA was a large increase, usually to more than double, of the dopamine content of the caudate nucleus. In three of the four experiments in which records were made of the EEG, there was arousal restricted to the side of the infusion. The noradrenaline in the hypothalamus and in the midbrain was unchanged, and thus it is tempting to attribute the arousal to the accumulation of dopamine in the caudate nucleus. That the caudate nuclei can exert an effect on the arousal mechanism follows from the work of HEUSER, BUCHWALD and WYERS (1961). These authors showed that electrical stimulation of the caudate nucleus at low frequencies produced spindles, whereas high frequencies caused desynchronization of the EEG. The slow onset of the response suggests that the DOPA itself is not responsible but a transformation product. This view is supported by the well-known fact that arousal by DOPA can be greatly facilitated by the administration of monoamine oxidase inhibitors, which favour the accumulation of amines. Yet, the question remains, whether the most obvious chemical change produced is also the one responsible for the arousal. It seemed particularly important to make sure what happened in the reticular formation itself. Only very small quantities of dopamine are normally found in those parts of the midbrain which contain the reticular formation. This amount did not increase to high concentrations after arterial infusion of DOPA, but the percentage increase was of the same order as that found in the caudate nucleus. If we consider the dopamine in the reticular formation entirely as a precursor to noradrenaline without any function of its own, this increase should not have any physiological effects since the noradrenaline content of the midbrain did not rise. If, however, dopamine is an active substance in its own right also in regions in which its concentration is low and noradrenaline is the dominant catecholamine, the possibility cannot be ruled out that a small increase at such a site has physiological effects and could possibly cause arousal.

## SUMMARY

Dihydroxyphenylalanine (5–20 mg) was infused into one cartoid artery of cats through a catheter tied into one lingual artery. When dilatation of the pupil (anaesthetized cats) or encephalographic arousal (*cerveau isolé* preparation) had developed on the side of the infusion, the brain was removed and catecholamine estimations were carried out in different regions of the two cerebral hemispheres.

The dopamine content of the caudate nucleus was increased on the side of the infusion, often by more than 100%.

The dopamine content of hypothalamus and midbrain-reticular formation of the treated side was increased by a similar proportion.

The noradrenaline content of the hypothalamus and of the midbrain was not raised by the infusion.

The possible relationship of chemical and physiological changes elicited by the infusion is discussed.

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# THE SUBCELLULAR LOCALIZATION OF DOPAMINE AND ACETYLCHOLINE IN THE DOG CAUDATE NUCLEUS

BY

**R. LAVERTY, I. A. MICHAELSON, D. F. SHARMAN and V. P. WHITTAKER**

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# THE SUBCELLULAR LOCALIZATION OF DOPAMINE AND ACETYLCHOLINE IN THE DOG CAUDATE NUCLEUS

R. LAVERTY, I. A. MICHAELSON*, D. F. SHARMAN AND V. P. WHITTAKER

*From the Biochemistry Department and the Pharmacology Unit, Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge*

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The distribution of dopamine (3,4-dihydroxyphenylethylamine) between the subcellular fractions from homogenates of dog caudate nucleus was compared with the distributions of acetylcholine, 5-hydroxytryptamine and lactate dehydrogenase activity. The distributions of noradrenaline and 5-hydroxytryptamine between the subcellular fractions from homogenates of dog hypothalamus were also determined. Most of the dopamine, in contrast to acetylcholine, occurred in the soluble supernatant fraction; the remainder was associated with the fractions rich in pinched-off nerve ending particles, but localization in any one fraction was not as sharp as that of acetylcholine. Evidence is presented that suggests that the dopamine occurs in a free or easily released form throughout cell cytoplasm.

In a study of the subcellular distribution of dopamine (3,4-dihydroxyphenylethylamine) in the brain stem of the rabbit, Weil-Malherbe & Bone (1957b, 1959) found that after high speed centrifugation the amine was equally distributed between the particulate material and the supernatant fractions and that approximately 40% of the total dopamine in the brain stem was associated with that fraction of the particulate material which contained the mitochondria. More recently, Bertler, Hillarp & Rosengren (1960), using caudate nuclei from cats and rabbits, and Weil-Malherbe, Posner & Bowles (1961), using rabbit brain, have found that after subcellular fractionation only 25% of the dopamine was recovered in the particulate material so that the majority of the dopamine seemed to occur in the "cytoplasmic sap." A cytoplasmic localization for dopamine was also found in bovine splenic nerves by Schümann (1958).

Originally, it was thought that the amines acetylcholine and 5-hydroxytryptamine found in the central nervous system were associated with subcellular structures similar to or identical with mitochondria (Bodian, 1942; Brodkin & Elliott, 1953; Walaszek & Abood, 1957). More recent work using techniques of differential and density gradient centrifugation have demonstrated that acetylcholine (Hebb & Whittaker, 1958; Whittaker, 1959), 5-hydroxytryptamine (Whittaker, 1959; Michaelson & Whittaker, 1963) and noradrenaline (Chruściel, 1960; Michaelson, unpublished) are associated with a distinct subfraction of brain homogenates which

* Present address: Laboratory of Chemical Pharmacology, National Heart Institute, N.I.H., Bethesda 14, Maryland, U.S.A.

has been shown by electron microscopy to consist mainly of pinched-off nerve ending particles (Gray & Whittaker, 1962); for these particles the name "synaptosome" has recently been adopted (Whittaker, Michaelson & Kirkland, 1963b).

The present investigation was undertaken to examine the subcellular localization of dopamine in the dog caudate nucleus and to obtain information as to the nature of the subcellular structure with which dopamine is associated. This region was selected because of its known high content of dopamine. A comparison was made with the subcellular distributions of acetylcholine and 5-hydroxytryptamine, also present in this tissue in relatively high concentrations. Lactate dehydrogenase was used as a cytoplasmic marker and comparisons were made with the distribution of noradrenaline in the hypothalamus of the dog.

## METHODS

### *Preparation of fractions*

Mongrel dogs anaesthetized with ether, chloroform or chloralose were bled from a carotid artery. The brain was removed and the caudate nuclei (and hypothalamus) were rapidly excised and weighed; the tissues were then homogenized in 0.32 M-sucrose solution using a glass and Perspex homogenizer (Aldridge, Emery & Street, 1960) to give a homogenate containing 10% (w/v) of tissue. A sample of homogenate was kept for analysis.

In initial experiments, this homogenate was separated into total particulate material ($P$) and high-speed supernatant fluid ($S$), by centrifugation at 100,000 $g$ for 1 hr in the AH 40 head of a Spinco Model L preparative ultracentrifuge. In other experiments the homogenate was separated initially into three primary particulate fractions essentially as described by Whittaker (1959). The first particulate fraction ($P_1$) was obtained by centrifuging for 11 min at 1,000 $g$ in a Servall bench centrifuge. This $P_1$ fraction was washed twice with 5 ml. of 0.32 M-sucrose solution and the washings were added to the supernatant fluid from the $P_1$ preparation. The next particulate fraction ($P_2$) was separated from the supernatant fluid of the $P_1$ preparation by centrifuging at 17,300 $g$ for 1 hr in a Servall RC-2 refrigerated automatic centrifuge. A further small particulate fraction ($P_3$) was separated from the supernatant fluid of the $P_2$ preparation by centrifugation at 100,000 $g$ for 1 hr in a Spinco Model L preparative ultracentrifuge, leaving a final high-speed supernatant fraction ($S_3$) containing the soluble constituents of the cell cytoplasm diluted with sucrose solution.

Subfractionation of the $P_2$ fraction by equilibrium density gradient centrifugation was carried out as described by Whittaker (1959). The $P_2$ pellet was resuspended in 0.32 M-sucrose solution so that 1 ml. of suspension corresponded to 500 mg of original tissue. The suspension (1 ml./tube) was layered on top of a discontinuous density gradient, prepared 1 hr before use, consisting of 2 ml. of 0.8 M-sucrose solution layered over an equal volume of 1.2 M-sucrose solution, and centrifuged at 100,000 $g$ for 1 hr in the SW 39 swing-out bucket head of the Spinco Model L preparative ultracentrifuge. Three distinct subfractions were obtained; the first ($A$) consisted of particles less dense than 0.8 M-sucrose solution, the second ($B$) of particles of density intermediate between 0.8 M- and 1.2 M-sucrose solution, and the third (C) of particles denser than 1.2 M-sucrose solution. All operations were carried out at 0° to 4° C.

### *Analysis of fractions*

Each primary particulate fraction was resuspended in 1 ml. of 0.32 M-sucrose solution. After removal of small samples for electron microscopy, acetylcholine assay and estimation of lactate dehydrogenase activity, each fraction was diluted to 2 ml. with 0.01 N-hydrochloric acid and the protein precipitated with 2.0 ml. of 0.8 N-perchloric acid. The $S_3$ fraction was adjusted to 0.4 N with respect to perchloric acid with 12 N-perchloric acid. The samples were then centrifuged and the acid supernatant fractions separated from their packed solids. The

perchloric acid was removed by neutralizing each of the acid supernatant fractions with 3 N-potassium carbonate solution to *p*H 4.0 and centrifuging in the cold.

Separation of the catechol amines by ion exchange column chromatography was carried out as described by Bertler, Carlsson & Rosengren (1958): the neutralized perchloric acid extracts were passed through a column of Dowex 50 X-8 (200–400 mesh, 3×25 mm) equilibrated with 1.0 N-sodium acetate buffer (*p*H 6). For extracts of caudate nuclei the column was washed with 6 ml. of 0.4 N-hydrochloric acid to remove any dihydroxyphenylalanine or noradrenaline, and the dopamine eluted with 8 ml. of 2 N-hydrochloric acid. For hypothalamic extracts the column was washed with 4 ml. of water and the noradrenaline eluted with 8 ml. of 0.4 N-hydrochloric acid.

Estimation of the dopamine was carried out fluorimetrically after condensation with ethylene diamine, as described by Weil-Malherbe & Bone (1952, 1957a). The fluorescence derived from the dopamine in the final eluate was compared in each case with the fluorescence produced by a standard amount of dopamine added to a fraction of the same eluate to ensure the correct development of the fluorescence. In control experiments the recovery of dopamine added to tissue samples was usually greater than 70%.

Because of the small amounts of tissue available, acetylcholine was assayed on the leech muscle micro-preparation described by Szerb (1961) using physostigmine (1 mg/100 ml.) as the anticholinesterase in the bathing fluid. The samples to be assayed were heated to 100° C at *p*H 4 to release bound acetylcholine and diluted with leech Locke solution before assay. In experiments in which the homogenization was carried out in the presence of physostigmine it was found that physostigmine was carried through the extraction procedure for dopamine and formed a highly fluorescent compound with ethylene diamine. Thus it was not possible to estimate the dopamine in these experiments.

The 5-OR indolyl compounds were measured as described by Ashcroft & Sharman (1962). Whenever possible, the compounds were separated into basic (presumably 5-hydroxytryptamine) and acidic (presumably 5-hydroxyindolylacetic acid) fractions. In the case of the primary particulate fractions ($P_1, P_2, P_3$), which were also assayed for dopamine, only a small quantity of the protein-free extract was available; this was analysed for total 5-OR indolyl compounds.

Estimation of noradrenaline was carried out fluorimetrically as described by Sharman, Vanov & Vogt (1963). Lactate dehydrogenase activity was measured spectrophotometrically as described by Johnson (1960) and is expressed as the change in extinction of the reaction mixture at 340 m$\mu$ ($\Delta E_{340}$) with time.

The concentration of amines (or enzyme) in the tissue fractions was expressed as m$\mu$moles (or $\Delta E_{340}$/min)/volume of fraction derived from 1 g of fresh tissue. The distribution of constituents between fractions was expressed as the percentage of the total recovered constituent found in each fraction. The results of similar experiments are expressed as the mean value and, with more than four observations, the standard deviation from the mean was calculated.

*Electron microscopy*

This was carried out by the negative staining method as described by Horne & Whittaker (1962). Preparations were fixed at 0° C by the addition of equal volumes of 10% (w/v) formaldehyde in 0.32 M-sucrose solution previously neutralized to *p*H 7.2 with 0.33 N-sodium hydroxide solution. The mixture was diluted with six times its volume of ice-cold 1% (w/v) aqueous phosphotungstic acid which had been brought to *p*H 7.2 with 2 N-sodium hydroxide and was then transferred to grids with a micropipette. Most of the droplet was removed from the grid with filter paper. A thin film of suspension remained which dried rapidly leaving particles embedded in solid sodium phosphotungstate. In this method particles are seen as a whole and not in section.

## RESULTS

### *Distribution between particulate-bound and soluble material*

A number of experiments were carried out in which an homogenate of dog caudate nuclei was separated into total particulate material (*P*) and a high-speed supernatant fluid (*S*) which were then analysed. The results for dopamine, 5-OR indolyl compounds, acetylcholine and lactate dehydrogenase activity are summarized in Table 1. It will be seen that dopamine resembles lactate dehydrogenase and

TABLE 1

DISTRIBUTION OF CONSTITUENTS OF DOG CAUDATE NUCLEUS HOMOGENATES BETWEEN THE TOTAL PARTICULATE MATERIAL (*P*) AND THE HIGH SPEED SUPERNATANT (*S*) FRACTION

The homogenate content is expressed in mμmoles/g of original tissue ; lactate dehydrogenase activity is expressed as $\Delta E_{340}$/min/g of original tissue. *These fractionation experiments were carried out using sucrose solution containing physostigmine sulphate (32·2 mg/l.) to preserve any free acetylcholine

| | No. of experiments | Mean homogenate content | Distribution (% of recovered activity) | |
|---|---|---|---|---|
| | | | *P* | *S* |
| Dopamine | 3 | 40 | 37 | 63 |
| Lactate dehydrogenase activity | 3 | 85 | 34 | 66 |
| Acetylcholine* | 2 | 17 | 81 | 19 |
| Basic 5-OR indolyl compounds (5-hydroxytryptamine) | 3 | 1·6 | 70 | 30 |
| Acidic 5-OR indolyl compounds (5-hydroxyindolylacetic acid) | 4 | 1·7 | 26 | 74 |

5-hydroxyindolylacetic acid in being recovered predominantly in fraction *S* representing the soluble constituents of the cell sap diluted with suspension medium. This is in contrast to the localization of acetylcholine and 5-hydroxytryptamine, which are recovered mainly in the total particulate fraction. It was also found that homovanillic acid, the major acid metabolite of dopamine in the dog caudate nucleus (Sharman, 1963), occurred predominantly in the high-speed supernatant fraction.

### *Distribution between particulate fractions*

The results of analyses of the primary particulate fractions separated from dog caudate nucleus and dog hypothalamus are shown in Table 2. Since most of the acidic 5-OR compounds present is found in the high speed supernatant fluid (Table 1), the analyses of the total 5-OR compounds given in this section will represent predominantly the basic 5-OR compounds, that is 5-hydroxytryptamine.

It will be seen from Table 2A that the distribution of acetylcholine among the primary particulate fractions from dog caudate nucleus differed appreciably from that of dopamine, the acetylcholine being much more sharply localized in the $P_2$ fraction.

In the experiments analysing the subcellular distribution of noradrenaline and 5-OR indolyl compounds in dog hypothalamus it was found that both amines were principally associated with the $P_2$ fraction (Table 2B). It is of interest to note that the ratio of the total particulate-bound material to soluble material for dopamine

TABLE 2

PERCENTAGE DISTRIBUTION OF DOPAMINE, ACETYLCHOLINE, 5-OR INDOLYL COMPOUNDS, NORADRENALINE AND LACTATE DEHYDROGENASE ACTIVITY BETWEEN THE PRIMARY FRACTIONS FROM DOG CAUDATE NUCLEUS AND HYPOTHALAMUS HOMOGENATES

The homogenate content is expressed in mμmoles/g of original tissue ; lactate dehydrogenase activity is expressed in $\Delta E_{340}$/min/g of original tissue. Values are means and standard deviations.

| | No. of experiments | Homagenate content | Ratio of total particulate bound material to material in supernatant ($P_1+P_2+P_3:S_3$) | Percentage of total particulate bound material recovered in $P_1$ | $P_2$ | $P_3$ | Percentage recovery of homogenate content in combined fractions |
|---|---|---|---|---|---|---|---|
| A. *Dog caudate nucleus* | | | | | | | |
| Dopamine | 8 | 44± 7 | 41 : 59 | 33±11 | 54±11 | 13± 6 | 80±17 |
| Total 5-OR indolyl compounds | 7 | — | — | 27± 5 | 53±11 | 20±10 | — |
| Acetylcholine | 5 | 19± 3 | 95 : 5 | 9± 2 | 86± 6 | 5± 3 | 88± 9 |
| Lactate dehydrogenase activity | 2 | 100 | 51 : 49 | 27 | 67 | 6 | 89 |
| Acidic 5-OR indolyl compounds | 1 | — | — | 23 | 70 | 7 | — |
| B. *Dog hypothalamus* | | | | | | | |
| Noradrenaline | 3 | 7 | 68 : 32 | 10 | 64 | 26 | 90 |
| Total 5-OR indolyl compounds | 3 | — | — | 16 | 67 | 17 | — |

in caudate nuclei is the inverse of that for noradrenaline in the hypothalamus. In the particulate fractions from both tissues the 5-hydroxytryptamine distribution parallels the distribution of the corresponding catechol amine.

Subfractionation of the $P_2$ fraction by density gradient centrifugation led to a further distinction between dopamine and acetylcholine (Table 3). It was found that the acetylcholine was again more sharply localized, in this case in the *B* sub-fraction.

### *Electron microscopy*

Electron microscopic examination of the various fractions from the caudate nucleus showed that these were similar in composition to, but more heterogeneous than, the whole guinea-pig brain preparations previously examined by Gray & Whittaker (1962) and Horne & Whittaker (1962). Besides nuclei, myelin and fragments of incompletely homogenized tissue, the $P_1$ fraction contained free mitochondria, synaptosomes and many small membrane fragments. Some of the synaptosomes were very large and so would have been expected to sediment in this fraction. This has been observed with the large synaptosomes formed from mossy fibre endings in homogenates of cerebellar cortex (Whittaker, 1962).

The $P_2$ fraction contained, as with whole guinea-pig brain preparations, myelin fragments, mitochondria, synaptosomes and many small membrane fragments mostly oval or circular in outline, down to microsomal dimensions. The synaptosomes varied greatly in size. Subfraction *A* of $P_2$ consisted mainly of myelin and small membrane fragments; however, synaptosomes were also present in considerable numbers. Subfraction *B* was a relatively homogeneous fraction

TABLE 3

PERCENTAGE DISTRIBUTION OF DOPAMINE, ACETYLCHOLINE AND LACTATE DEHYDROGENASE ACTIVITY AMONG SUBFRACTIONS OF THE $P_2$ PRIMARY FRACTION

Values are means with standard deviations.

| | Dopamine | Acetylcholine | Lactate dehydrogenase | Particles identified in the electron microscope |
|---|---|---|---|---|
| No. of experiments | 5 | 4 | 2 | |
| % in fraction *A* | 45± 7 | 19 | 33 | Myelin, small membrane fragments, many small synaptosomes |
| % in fraction *B* | 47±14 | 79 | 56 | Mainly synaptosomes, some shrunken |
| % in fraction *C* | 8± 7 | 2 | 11 | Mainly mitochondria, a few shrunken synaptosomes |
| Content of $P_2$/g of original tissue | 7·8±1·9 mμmoles | 15 mμmoles | $\Delta E_{340}^{30}$/min | |
| % recovery of $P_2$ content in combined fractions | 70±19 | 81 | 95 | |

consisting mainly of synaptosomes, many large (2 μ or more in diameter), often with considerable lengths of axons attached, others shrunken and bizarre in shape and densely packed with vesicles. This shrinking was caused by exposure to hypertonic sucrose solution. Noteworthy was the almost complete absence of free mitochondria in *A* and *B*, though small mitochondria were frequently present inside synaptosomes. By contrast, the *C* subfraction consisted almost entirely of mitochondria, often morphologically abnormal as previously observed in positively stained thin sections of the corresponding whole guinea-pig brain fraction. Occasional particles in this fraction poorly penetrated by phosphotungstate and often possessing long axon-like " tails " were tentatively identified as synaptosomes whose cytoplasm had fused to a dense mass as a result of dehydration in hypertonic sucrose solutions (the " black bodies " in the positively stained preparations of Gray & Whittaker, 1962).

Fraction $P_3$ consisted mainly of vesicular membrane fragments (microsomes) of varying size from about 0.02 to 0.07 μ ; there were also a few small synaptosomes and mitochondria. Two fractions obtained by subfractionating $P_3$ on a density gradient did not differ except that the smaller particles were more plentiful in the light fraction and the synaptosomes and the mitochondria were recovered with the larger membrane fragments in the denser fraction.

## DISCUSSION

### *Dopamine*

The topographical distribution of dopamine among the various parts of the brain (Bertler & Rosengren, 1959) does not correspond to that of noradrenaline (Vogt, 1954). It is possible that dopamine ocurs in the brain other than as a precursor of noradrenaline. Information about the subcellular distribution of dopamine in brain tissue might help in elucidating its function.

In the present study the subcellular distribution of dopamine in the caudate nucleus of the dog between total particulate and high-speed supernatant fractions was somewhat similar to that observed by Weil-Malherbe & Bone (1959) using rabbit brain stem, but it differed from the distributions of acetylcholine and

5-hydroxytryptamine in the caudate nuclei fractions, and also from that of noradrenaline in dog hypothalamus homogenates. The distribution of these latter amines in dog tissue corresponds well, however, with the distributions of acetylcholine (Whittaker, 1959), 5-hydroxytryptamine (Michaelson & Whittaker, 1963) and noradrenaline (Michaelson, unpublished observations) in guinea-pig whole brain tissue. The dopamine distribution between total particulate and supernatant fractions was closely paralleled by the distribution of lactate dehydrogenase activity, and was also matched by the distribution of 5-hydroxyindolylacetic acid. This difference in distribution between dopamine and the other amines must reflect a difference in the subcellular localization or metabolism of dopamine when compared with the other amines.

The percentage distribution of dopamine in the three primary particulate fractions from caudate nuclei is similar to that found for 5-hydroxytryptamine in this tissue, both being less sharply localized than acetylcholine. Dopamine from caudate nucleus homogenates, unlike 5-hydroxytryptamine in whole guinea-pig brain (Michaelson & Whittaker, 1963), is not readily lost from the particulate material during the prolonged manipulation in sucrose media involved in the fractionation procedure, as shown by the similarity in the ratios $P/S$ (Table 1) and $(P_1+P_2+P_3)/S_3$ (Table 2).

Upon subfractionation of the "crude mitochondrial" ($P_2$) fraction by density gradient centrifugation dopamine was not as closely associated with any one subfraction as was observed for acetylcholine. In the present study dopamine was equally distributed between subfractions $A$ and $B$ whereas acetylcholine was preferentially localized in $B$. It would appear then that the structures containing dopamine in the dog caudate nucleus have a larger range of densities than those associated with acetylcholine. Electron microscopic examination of the subfractions from the dog caudate nucleus revealed that the density gradient separation failed to resolve the $P_2$ fraction into such relatively homogeneous subfractions as was observed for guinea-pig brain (Gray & Whittaker, 1962). Both the $A$ and $B$ subfractions of $P_2$ contained many pinched-off nerve endings. It would appear that the synaptosomes in the dog caudate nucleus have a larger range of sizes than the synaptosomes derived from guinea-pig brain tissue.

Lactate dehydrogenase activity and dopamine had similar relative distributions. Lactate dehydrogenase is thought to be associated with the cell cytoplasm (Johnson, 1960; Johnson & Whittaker, 1963). This association suggests that dopamine is also distributed through the cell cytoplasm and that the dopamine associated with the particulate material is trapped in the cytoplasm of the pinched-off nerve ending particles. Additional evidence for a cytoplasmic localization of dopamine came from two preliminary experiments in which the particulate fractions containing dopamine were subjected to water-treatment and density gradient separation (Whittaker *et al.*, 1963a, b). After water-treatment 80% of the particulate-bound dopamine was recovered in the supernatant fraction. It appears that the dopamine is contained in a loosely bound or free form in a subcellular particle that can be disrupted by water-treatment. In contrast, acetylcholine was still associated with particulate material after water-treatment.

*Acetylcholine*

The homogenates of caudate nuclei contained a relatively high concentration of acetylcholine. Even in the presence of physostigmine, which preserves any free acetylcholine initially present, there was very little acetylcholine in the high-speed supernatant fluid. Thus nearly all of the acetylcholine was associated with particulate material. On fractionation, this particle-bound acetylcholine was localized in the $P_2$ fraction and the *B* fraction derived from it, in spite of the presence of considerable numbers of synaptosomes in the $P_1$ and *A* fractions. It is not known for certain whether the free acetylcholine in brain tissue homogenates represents acetylcholine released from cholinergic nerve endings which have been disrupted during homogenization or acetylcholine originally present in other parts of the cholinergic neurone. The latter appears probable, since Hebb & Whittaker (unpublished) found that the acetylcholine in homogenates of ventral spinal columns, a region in which cholinergic neurones are represented mainly by the cell bodies of motor neurones and in which the only known cholinergic endings are those of the recurrent collateral fibres to Renshaw cells, was mainly in the free form. If this interpretation is correct, the results with caudate nuclei strongly imply that cholinergic neurones are here represented almost exclusively by endings which break off on homogenization to form a rather uniform population of synaptosomes containing acetylcholine. This assumption is supported by the recent work of Shute & Lewis (1963) who demonstrated histochemically in the rat that there is a dense cholinergic neuropil in the caudate nucleus and putamen contributed by the striatal radiation from the ventral midbrain tegmentum. Comparisons between the morphology of the synaptosomes of fraction *B* and the cholinergic neuropil are clearly needed.

It may be concluded from these experiments that the subcellular particle with which part of the dopamine is associated is similar to but distinguishable from that with which acetylcholine is associated. It was not possible to separate particles containing dopamine and 5-hydroxytryptamine. However, the distribution ratios of the latter two amines between the particulate and high-speed supernatant fractions are quite different. A possible explanation is that both these amines are in the same cell but that 5-hydroxytryptamine is confined to the synaptosomes and that dopamine is distributed throughout the cell. It cannot be excluded, however, that dopamine and 5-hydroxytryptamine are in different cells.

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# SHORT COMMUNICATIONS

## Localization of acetylcholine, 5-hydroxytryptamine and noradrenaline within subcellular particles derived from guinea pig subcortical brain tissue

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WHEN brain tissue is homogenized in 0·32 M sucrose, nerve endings escape disruption and are snapped off to form nerve-ending particles (NEPs) which can be isolated in a discrete fraction by differential

and density gradient centrifugation.[1–3] On suspension in water, the outer membranes of the NEP are disrupted, and soluble cytoplasmic constituents together with some synaptic vesicles are released. A simple density gradient procedure[4,5] permits the separation of fractions: fraction *O* containin soluble cytoplasm; *D*, synaptic vesicles; *E*, microsomes; *F* and *G*, membrane fragments and NEI ghosts; *G* and *H*, partially ruptured NEPs and *I*, mitochondria. Bound acetylcholine was found to b bimodally distributed with peaks in the synaptic vesicle (*D*) and damaged NEP (*H*) fractions.

Other work[6–8] has shown that 5-hydroxytryptamine (HT) and noradrenaline (NA) are also local ized in the NEP fraction (although not so sharply as acetylcholine). The object of the present work wa to find out whether these two amines behaved similarly to acetylcholine on hypotonic disruption and in particular whether a synaptic vesicle fraction containing them could be isolated.

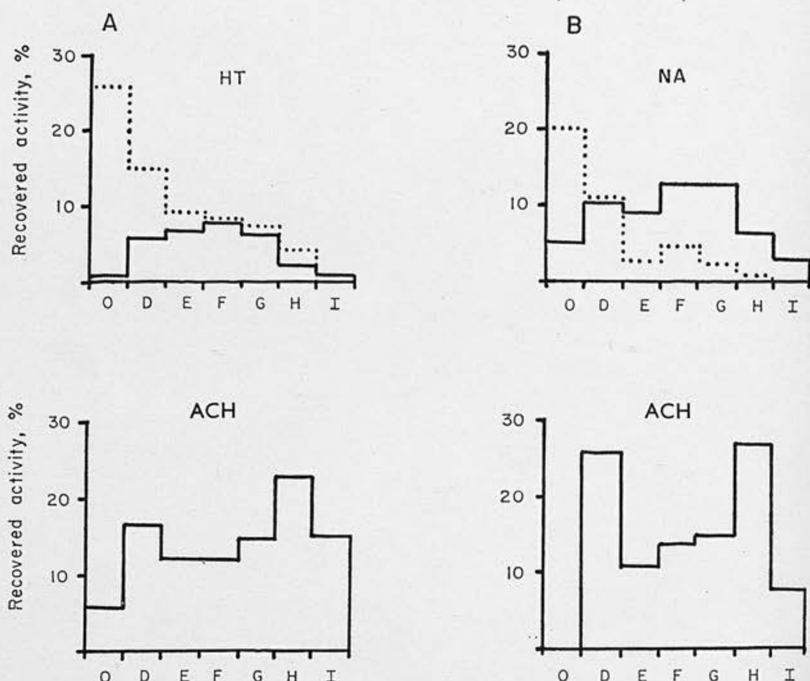


Fig. 1. Comparison of the distribution within a density gradient of (A) 5-hydroxytryptamine (HT) and acetylcholine (ACh), and, (B) noradrenaline (NA) and acetylcholine (ACh) derived fro hypotonically-treated subcellular particles from guinea pig subcortical brain tissue. Solid lines repr sent bound amine, dotted line represents free amine. Ordinate: percentage in each fraction of the tot amine in the gradient. Amine recovered, as mμmole/g tissue processed (no. of experiments averag and percentage recovery in brackets): HT, 1·27 (3, 137); ACh, 0·82 (2, 66); NA, 1·1 (2, 123); ACh 5·8 (3, 132).

Some technical difficulties were encountered. The estimation of HT as total 5-OR indolyl compound without solvent extraction as used earlier[4] gave falsely high values with fractions from the low part of the density gradient owing to the presence of interfering substances in the sucrose. This w overcome by solvent extraction into butanol.[9] The levels of the amines, particularly of HT, in who brain preparations were low. The amines liberated as the free form on hypotonic rupture are n destroyed, as with acetylcholine, so that means had to be devised for separately estimating free an bound amines in each fraction. Accordingly our earlier procedure was modified and two kinds preparation were used.

In the experiments with HT, the level of the amines was raised by injecting iproniazid (100 mg/k subcutaneously 14–20 hr before killing the animals. This procedure does not alter the amine distrib tion in the primary fractions.[6] The midbrain, diencephalon and caudate nuclei, the regions richest HT, were homogenized. After removal of the nuclear ($P_1$) fraction at 1000 $g$ for 11 min, a cru

mitochondrial ($P_2$) fraction was prepared by centrifuging the homogenate at 17000 *g* for 60 min. The $P_2$ fraction was suspended in water and separated into fractions *O–I* as previously described.[4] Each fraction was diluted with water and centrifuged at 100,000 *g* for 60 min to give supernatants and pellets. The amine present in the supernatants is regarded as "free" and that in the pellets as "bound". All the acetylcholine, as estimated by the leech muscle micromethod[10] after release by heating at 100° for 10 min at pH 4,[11] was recovered in the pellets, i.e. was "bound", since in the absence of an anticholinesterase any free acetylcholine would be destroyed.

In the NA experiments, there was no premedication; only the midbrain and diencephalon were used, since the caudate nucleus is known to contain very little of this amine. The NA was estimated fluorimetrically.[12] Distribution studies with the primary fractions from these tissues showed that about 30 per cent of the NA was recovered in the microsomal ($P_3$) fraction; accordingly, after separation of the $P_1$ fraction, a combined $P_2$ and $P_3$ fraction was prepared by centrifuging at 100,000 *g* instead of 17,000 *g* for 1 hr. Disruption in water was carried out more vigorously than in the HT series, with mechanical homogenization for about 30 sec. Morphological examination showed that fractions *D–F* were less homogeneous than the corresponding fractions from whole brain; they all contained synaptic vesicles heavily contaminated with microsomes.

The results of the two series of experiments are shown in Fig. 1. In the HT series (Fig. 1A) bound acetylcholine was bimodally distributed with peaks in *D* and *H*. The *D* fraction was less opaque and the yield of vesicular acetylcholine was lower than in previous whole brain studies.[4] (Dog caudate nucleus preparations have also been found to be more resistant to hypotonic rupture than guinea-pig whole brain.) In contrast to acetylcholine, bound HT peaked in *F*, but was not clearly localized in any fraction and showed no evidence of bimodal distribution. About 70 per cent of the total HT recovered was present in the free form; fraction *O*, corresponding to soluble cytoplasm, contained the most, with decreasing amounts in the lower parts of the gradient. The free amine in the lower fractions could have occurred as the result of diffusion from *O*.

In the NA series (Fig. 1B), acetylcholine was again bimodally distributed, and perhaps because of the more vigorous disruption, the opacity of the *D* fraction and the yield of acetylcholine in this fraction was greater. A greater proportion of NA was present in the bound form than was found with HT, but the distribution of bound NA was similar to that of bound HT. The distribution of free NA was also similar to that of HT, the highest concentration being in *O* with decreasing amounts in lower fractions.

To summarize: when particulate fractions containing NEPs are disrupted by exposure to hypotonic conditions, a considerable proportion of free amine is liberated and is recovered in the soluble cytoplasmic (*O*) fraction, with decreasing amounts in lower fractions. In the case of NA, over half the amine remains bound, a proportion similar to that found with acetylcholine[11] and of this significant amounts are associated with particulate material free from NEPs. With HT, the proportion remaining bound is lower and the absolute amounts present are too low to permit firm conclusions as to the nature of the particulate binding sites.

It is obvious from these experiments that the storage sites for NA and HT within the subcellular particles differ from that for acetylcholine and require further characterization. However, it is not yet possible to distinguish between the localization of NA and HT.

In comparing the behaviour of NA and HT, the differences in the preparations used, the vigour with which they were disrupted and a possible selective effect of iproniazid in the localization of HT within the intact storage particle must be borne in mind. Further work is in progress to define more accurately the nature of the amine storage sites.

*A.R.C. Institute of Animal Physiology,*
*Babraham,*
*Cambridge.*

I. A. Michaelson*
V. P. Whittaker†
R. Laverty‡
D. F. Sharman

* Postdoctoral Fellow, National Heart Institute, United States Public Health Service. Present address: Laboratory of Chemical Pharmacology, National Heart Institute, N.I.H., Bethesda, Md.

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‡ Beit Memorial Research Fellow.

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# A FLUORIMETRIC METHOD FOR THE ESTIMATION OF 4-HYDROXY-3-METHOXYPHENYLACETIC ACID (HOMOVANILLIC ACID) AND ITS IDENTIFICATION IN BRAIN TISSUE

BY

D. F. SHARMAN

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# A FLUORIMETRIC METHOD FOR THE ESTIMATION OF 4-HYDROXY-3-METHOXYPHENYLACETIC ACID (HOMOVANILLIC ACID) AND ITS IDENTIFICATION IN BRAIN TISSUE

BY

D. F. SHARMAN

*From the Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge*

(*Received December* 7, 1962)

A fluorimetric method for the estimation of 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid) has been developed and applied to normal brain tissue. The presence of homovanillic acid in the caudate nucleus of normal animals of several species has been demonstrated.

Methods for the estimation of 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid) have been based on the formation of coloured compounds by coupling the acid with diazotized *p*-nitroaniline (Armstrong, Shaw & Wall, 1956) or diazotized sulphanilic acid (Shaw, McMillan & Armstrong, 1957 ; DeEds, Booth & Jones, 1957). The presence of atmospheric contaminants can interfere with these methods of estimation (Shaw & Trevarthen, 1958). Sweeley & Williams (1961) have used gas chromatography to separate and estimate homovanillic acid. Recently, Ruthven & Sandler (1962) have reported a method based on the conversion of homovanillic acid to 3,4-dihydroxyphenylacetic acid, which is then estimated colorimetrically.

Fluorimetric methods of estimation are, in general, more sensitive than colorimetric methods. Duggan, Bowman, Brodie & Udenfriend (1957) suggested that the fluorescence exhibited by homovanillic acid at *p*H 7 might form the basis of a chemical assay. Many other aromatic acids fluoresce in the same spectral region as homovanillic acid, and such a method might be criticized for its lack of specificity. This report is concerned with the development of a more specific fluorimetric method of determining homovanillic acid and with the identification of this compound as a normal constituent of brain tissue.

## METHODS

The phenol and indole derivatives used were:

Homovanillic acid (4-hydroxy-3-methoxyphenylacetic acid), 4-hydroxy-3-methoxyphenethylamine, normetanephrine [2-amino-1-(4-hydroxy-3-methoxyphenyl)ethanol] and metanephrine [1-(4-hydroxy-3-methoxyphenyl)-2-methylaminoethanol], each from the California Corporation for Biochemical Research ; 5-hydroxyindol-3-ylacetic acid, 4-hydroxyphenylacetic acid, 3-methoxyphenylacetic acid, 4-methoxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, 4-hydroxy-3-methoxycinnamic acid and 4-hydroxycinnamic acid, each from L. Light & Co. ;

4-hydroxy-3-methoxymandelic acid, 4-hydroxy-3-methoxyphenylalanine, bis(4-hydroxy-3-methoxyphenylglycol) piperazine salt and *N*-acetyl-4-hydroxy-3-methoxyphenethylamine, prepared by the method described by Goldstein & Musacchio (1962).

The reagents required were:

Ferric chloride as a 1% w/v solution in 0.1 N-HCl. Solutions of ferric chloride in water are also suitable, but the fluorescence developed from homovanillic acid was more consistent when aqueous solutions of ferric chloride had been left to stand for a few days. This was thought to be due to the presence of hydrochloric acid produced by hydrolysis.

Sodium hydroxide as a 20% w/v solution in freshly distilled water.

Both de-ionized and distilled water have been used, but a high blank fluorescence was observed with some samples of water, particularly samples which had been stored in polyethylene vessels. The most suitable water was freshly distilled water.

*Extraction of tissues.* Dogs, cats or rabbits were bled to death after anaesthetization with ether, chloroform or chloralose. The caudate nuclei were dissected out as rapidly as possible. The caudate nuclei of the cow and sheep were obtained immediately after slaughter with a captive bolt. Only undamaged tissues were used. The caudate nuclei were weighed and then homogenized in 2.0 ml. of 0.1 N-HCl per g of tissue. When immediate homogenization was not possible the tissues were frozen and kept at $-17°$ C. The acid homogenate was diluted with water to a volume of 10.0 ml./g of tissue and deproteinized by the addition of 2.0 ml. of a 10% w/v aqueous solution of zinc sulphate and 0.2 ml. of the 20% w/v solution of sodium hydroxide for each 10.0 ml. of the diluted homogenate. The homogenate was carefully mixed after each addition. The mixture was centrifuged at 900 *g* for 5 min and the supernatant filtered through Whatman no. 54 filter paper. The solution was adjusted to *p*H 1–2 (indicator paper) with concentrated hydrochloric acid, the solution saturated with sodium chloride and extracted by shaking with an equal volume of ethyl acetate for 5 min. A second extraction with an equal volume of ethyl acetate was carried out and the two extracts were combined and dried over anhydrous sodium sulphate for 2 to 24 hr at 4° C. The extract was then evaporated at 35° C to approximately 0.2 ml. under a stream of nitrogen. This concentrated extract was applied to paper for chromatographic development.

*Paper chromatography.* Two-dimensional chromatograms were developed on Whatman no. 1 paper which had been washed with 0.01 N-HCl. One-dimensional chromatograms were developed on Whatman no. 50 or Whatman no. 1 paper.

In those experiments in which portions of chromatograms were eluted and the eluates tested for the development of a fluorescence with the ferric chloride and sodium hydroxide solutions, the paper was treated to reduce the fluorescence derived from the paper itself. Whatman no. 50 paper was soaked in 2 N-NaOH for 24 to 48 hr. It was then washed by repeated changes of distilled or de-ionized water until no alkali was detected in the washings. The paper was stored under distilled water, which had been tested for a low fluorescence on adding the sodium hydroxide solution. Before use the paper was air-dried at 25° C. The one-dimensional chromatograms were developed on a modified form of the wick-and-strip chromatogram described by Mathias (1954). A typical shape is shown in Fig. 5. This method produces narrow bands across the chromatogram and the area of a "spot" is kept to a minimum.

The following solvent systems were used to develop the chromatograms.

System A: Isopropanol, ammonia solution and water (80:2:18).

System B: Benzene, propionic acid and water. For this system one can use either the organic phase of a 100:25:25 mixture or the single phase obtained by mixing the components in the proportions 100:70:4.

System C: 0.01 N-HCl saturated with sodium chloride.

System D: Butanol, water, glacial acetic acid and dichloroethane. The organic phase from a 1:1:1:3 mixture was used.

Two-dimensional chromatograms were developed for 15 hr with system A, dried in air and then developed for 7 hr with system B in a direction at a right-angle to that of the first development. The ascending technique was used in every instance.

Phenolic compounds were located on the chromatogram by spraying with a 1% w/v solution of Brentamine Fast Red GG Salt (I.C.I. Ltd.) in 0.1 N-HCl. This is a stabilized preparation of diazotized *p*-nitroaniline. The chromatogram was air-dried and then sprayed with a 5% w/v solution of sodium carbonate. The elution of portions of chromatograms was carried out with 0.01 N-HCl or with distilled water.

Concentrations of reagents expressed as percentages refer to w/v solutions.

## RESULTS

*The characterization of the fluorescence developed from homovanillic acid.* The method of estimation is based on the observation that a blue fluorescence develops when a solution of homovanillic acid is treated with ferric chloride and then made alkaline with sodium hydroxide. A known amount of homovanillic acid was placed in a 1.0 ml. glass-stoppered, volumetric flask of test-tube shape, and diluted to 1.0 ml. with water. The ferric chloride and sodium hydroxide solutions were added in the quantities given below. The flask was then centrifuged for 30 sec to remove the ferric hydroxide which had formed. The clear supernatant was transferred to a quartz cuvette and the fluorescence was examined in an Aminco-Bowman spectrophotofluorimeter. The wavelength of maximum activation was 305 mμ, and there was a smaller peak at 250 mμ. Maximum fluorescence was at a wavelength of 430 mμ. These are values uncorrected for instrumental error.

*The effect of light on the reaction.* Exposure of the reaction mixture to different intensities of light resulted in the development of different intensities of fluorescence. In order to obtain reproducible conditions, all reactions have been carried out in shade. The volumetric flasks were placed up to their rims in holes, drilled in small blocks of wood, before adding the reagents.

*The time of reaction with ferric chloride.* Samples containing 1.0 μg of homovanillic acid were mixed with 0.1 ml. of the ferric chloride solution and the reaction was allowed to proceed for different times ; 0.1 ml. of the sodium hydroxide solution was then added and the fluorescence (activation wavelength 305 mμ ; fluorescence wavelength 430 mμ) measured after removal of the ferric hydroxide. The effect of increasing the time of reaction is shown in Fig. 1. Maximal fluorescence was obtained when the reaction was allowed to proceed for 45 sec.

*The effect of the concentration of ferric chloride in the reaction mixture.* The reaction was carried out with 0.5 μg samples of homovanillic acid, using increasing amounts of ferric chloride. The results are shown in Fig. 2. The maximum fluorescence was developed with 1.0 mg of ferric chloride.

*The effect of the concentration of sodium hydroxide in the reaction mixture.* The effect of increasing the amount of sodium hydroxide on the fluorescence developed was investigated using 1.0 μg samples of homovanillic acid ; 0.1 ml. of the ferric chloride solution was allowed to act for 45 sec before adding the sodium hydroxide solution. Maximum fluorescence was obtained with 10 to 30 mg of sodium hydroxide (Fig. 3).

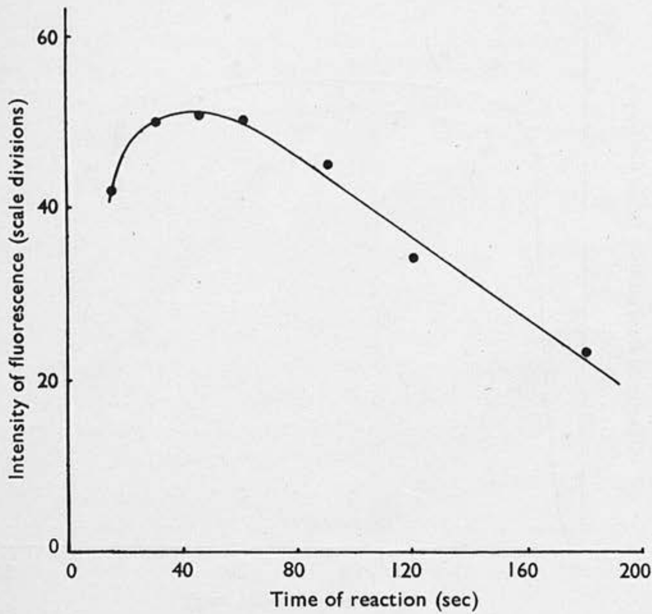


Fig. 1. The effect of the time of reaction (abscissa) with ferric chloride on the development of the fluorescence (ordinate) from homovanillic acid.

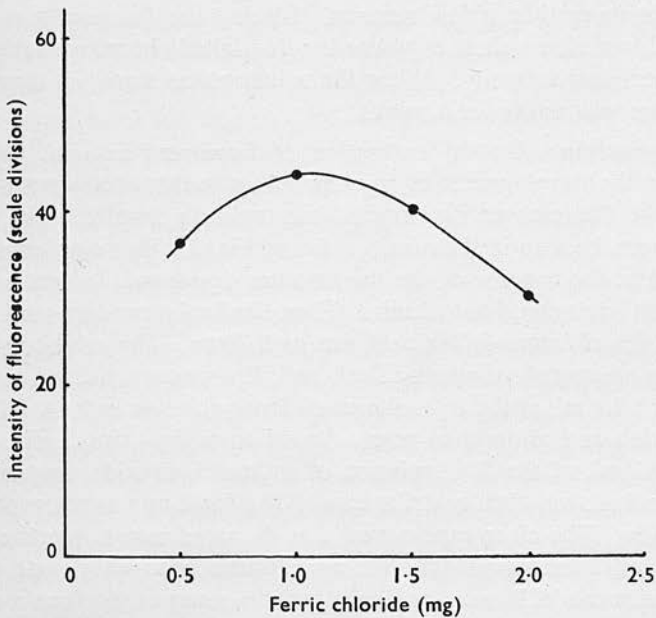


Fig. 2. The effect of the concentration of ferric chloride (abscissa, expressed as mg added) on the development of the fluorescence (ordinate) from homovanillic acid. The ferric chloride was allowed to react for 45 sec.

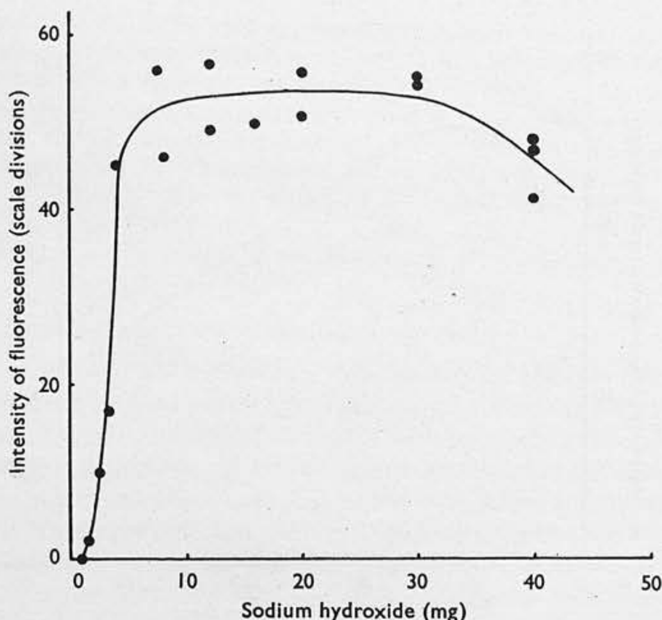


Fig. 3. The effect of the concentration of sodium hydroxide (abscissa, expressed as mg added) on the development of the fluorescence (ordinate) from homovanillic acid.

*The stability of the fluorescence.* The fluorescence faded on exposure to ultraviolet light for more than a few seconds. Placing the fluorescent solution in the dark for 1 to 2 min after a short exposure to ultraviolet light restored the fluorescence almost to its original intensity. When the solution was stored in the dark at 4° C the fluorescence was stable for a week.

*The final procedure for the estimation of homovanillic acid.* When all the conditions for the development of maximal fluorescence were present, there was variation in the fluorescence developed from replicate samples. An indication of this variation can be seen in the results given in Fig. 3. However, consistent results were obtained at the expense of the fluorescence developed, by increasing the time of reaction with ferric chloride to 2 min. Thus, the final procedure used in estimating the concentration of homovanillic acid was as follows. The solution was placed in a 1.0 ml. glass-stoppered volumetric flask and, if necessary, diluted to 1.0 ml. with distilled water ; 0.1 ml. of the 1% solution of ferric chloride in 0.1 N-HCl was mixed with the solution and allowed to react, shaded from light, for 2 min. At the end of this time 0.1 ml. of the 20% solution of sodium hydroxide was mixed with the solution. The flask was then centrifuged at 900 *g* for 2 min to remove the insoluble ferric hydroxide. The clear supernatant was decanted into a quartz cuvette. The fluorescence (activation wavelength 305 m$\mu$ ; fluorescence wavelength 430 m$\mu$) was measured at intervals of 30 sec for 2 min and the mean of the four readings taken. The solution was not exposed to the activating light for longer than 5 sec at a time.

This method was applied to different amounts of homovanillic acid. The results obtained on two occasions are shown in Fig. 4. The spectrophotofluorimeter was

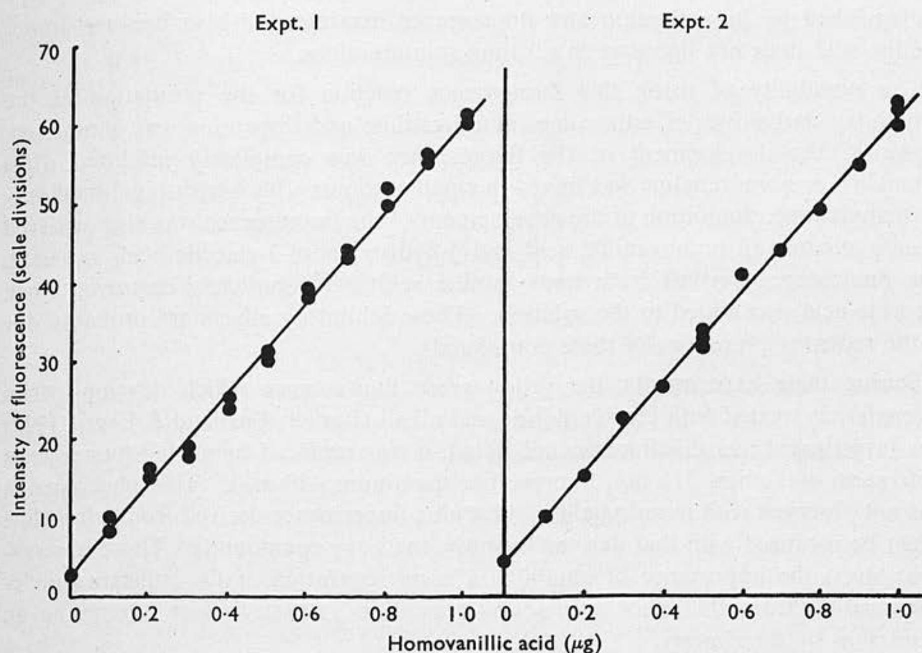


Fig. 4. The relationship between the amount of homovanillic acid (abscissa) and the fluorescence developed (ordinate), for two experiments.

standardized each time against the fluorescence of a block of Araldite CY212 epoxy resin, which enabled the same sensitivity to be obtained in the two experiments. Fig. 4 shows that there is a linear relationship between the amount of homovanillic acid in the sample and the fluorescence developed. The results are reproducible when the same reagents are used, but changes in the slope of the standard curve have been observed with different solutions of ferric chloride.

*The specificity of the method.* The reaction was applied to other organic acids and to some compounds structurally related to homovanillic acid. Of the compounds tested, 4-hydroxy-3-methoxymandelic acid, 4-hydroxy-3-methoxyphenethylamine, *N*-acetyl-4-hydroxy-3-methoxyphenethylamine, normetanephrine, metanephrine, 4-hydroxy-3-methoxyphenylalanine and 4-hydroxy-3-methoxyphenylglycol yielded a fluorescence with the same maximum as, and of somewhat less intensity than, that developed with homovanillic acid. 4-Hydroxy-3-methoxybenzoic acid (vanillic acid) and 4-hydroxy-3-methoxycinnamic acid gave rise to a very weak fluorescence, approximately 3% of that derived from homovanillic acid. No significant fluorescence was developed with 3-methoxyphenylacetic acid, 4-methoxyphenylacetic acid, 4-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid and 5-hydroxy-indol-3-ylacetic acid.

Serious interference might be caused by 4-hydroxycinnamic acid, which exhibits a blue fluorescence (maximum activation wavelength 350 m$\mu$, maximum fluorescence wavelength 450 m$\mu$) in alkaline solution. This fluorescence may, however, be

distinguished by its activation and fluorescence maxima and also because homovanillic acid does not fluoresce in alkaline solution alone.

The possibility of using this fluorescence reaction for the estimation of the 3-methoxy derivatives of adrenaline, noradrenaline and dopamine was considered. However, the development of the fluorescence was completely inhibited when adrenaline or noradrenaline was mixed in equal amounts with 4-hydroxy-3-methoxyphenethylamine. Inhibition of the development of the fluorescence was also observed when a mixture of homovanillic acid and 5-hydroxyindol-3-ylacetic acid was used. The fluorescence derived from homovanillic acid was completely destroyed when ascorbic acid was added to the solution. These inhibitory effects are probably due to the reducing properties of these compounds.

During these experiments, the yellow-green fluorescence which develops when adrenaline is treated with ferric chloride and alkali (Barker, Eastland & Evers, 1932) was investigated ; as this fluorescence faded, it was replaced by a blue fluorescence (activation maximum 315 m$\mu$, fluorescence maximum 450 m$\mu$). This phenomenon was not observed with noradrenaline. This blue fluorescence derived from adrenaline might be confused with that derived from the methoxy compounds. These observations stress the importance of obtaining a good separation of the substance under investigation from the other substances present in extracts before attempting an estimation by fluorimetry.

*The use of the reaction as a localizing test on paper chromatograms.* Paper chromatograms of authentic homovanillic acid and 4-hydroxy-3-methoxymandelic acid were sprayed with a 1% solution of ferric chloride in 0.1 N-HCl. When dried, the chromatograms were sprayed with a 20% solution of sodium hydroxide. Examination in ultraviolet light (Chromatolite ; Chance OX7 filter) revealed blue fluorescent spots at $R_F$ values corresponding with those of the two acids. The limit of detection was about 5 $\mu$g.

*The identification of homovanillic acid in brain tissue.* Dog caudate nucleus (13.2 g) and cow caudate nucleus (7.7 g) were extracted and the extracts subjected to two-dimensional chromatography. Table 1 gives the $R_F$ values and the colour reaction with Brentamine Fast Red GG of the main phenolic substance detected on the chromatograms ; a substance with an $R_F$ value in two solvent systems and a

TABLE 1

$R_F$ VALUES OF THE PHENOLIC SUBSTANCE ISOLATED FROM THE BRAIN OF DIFFERENT ANIMALS COMPARED WITH HOMOVANILLIC ACID CHROMATOGRAPHED UNDER THE SAME CONDITIONS

| | $R_F$ value in solvent system | | | | Colour with diazotized |
|---|---|---|---|---|---|
| | A | B | C | D | *p*-nitroaniline |
| Dog brain | 0·37 | 0·78 | 0·63 | 0·90 | Grey-blue |
| Homovanillic acid | 0·37 | 0·79 | 0·64 | 0·91 | Grey-blue |
| Cow brain | 0·35 | 0·76 | — | — | Grey-blue |
| Homovanillic acid | 0·36 | 0·78 | — | — | Grey-blue |
| Rabbit brain | — | 0·76 | — | — | Grey |
| Homovanillic acid | — | 0·76 | — | — | Grey |
| Cat brain | — | 0·64 | — | 0·91 | Grey-blue |
| Homovanillic acid | — | 0·64 | — | 0·91 | |

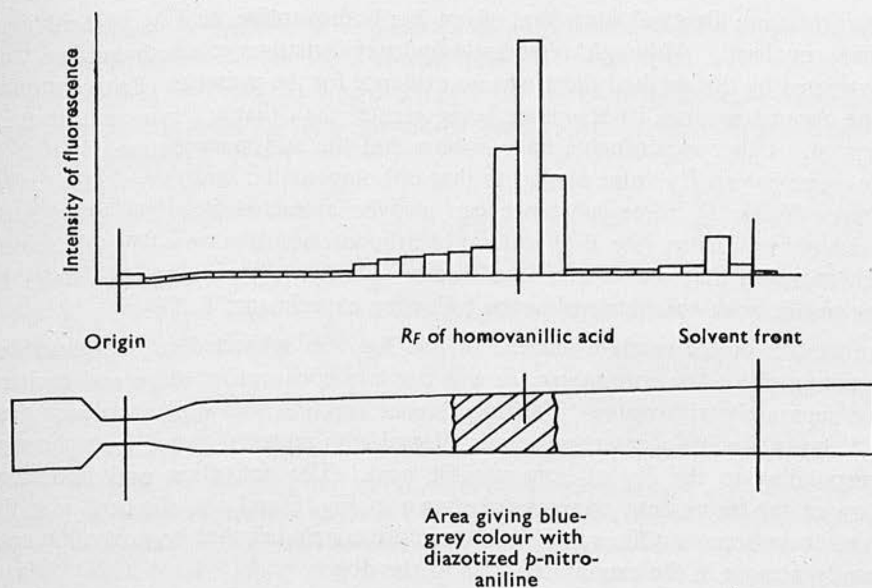


Fig. 5. The localization of apparent homovanillic acid on a chromatogram of an extract of the caudate nucleus of the dog.

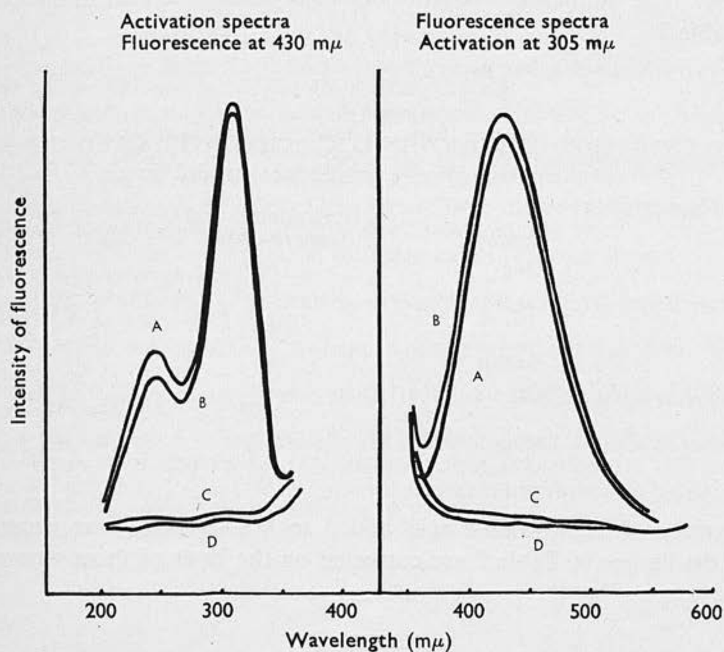


Fig. 6. Activation (left) and fluorescence (right) spectra of the fluorescence developed from the phenolic substance extracted from the caudate nucleus of the dog. A: substance extracted from the caudate nucleus. B: 2 μg of homovanillic acid. C: extract blank. D: reagent blank.

colour reaction identical with that given by homovanillic acid is present in the caudate nucleus. Although *N*-acetyl-4-hydroxy-3-methoxyphenethylamine would be extracted by this method there was no evidence for the presence of this compound on the chromatograms, its $R_F$ value being greater than that of homovanillic acid in system A. Other experiments have shown that the substance extracted from dog brain tissue has an $R_F$ value similar to that of homovanillic acid in two other solvent mixtures (Table 1). The substance can also be extracted from the ethyl acetate extracts of brain tissue into 10% sodium bicarbonate solution or N-$NH_4OH$ solution, which indicates that the substance is acidic. Further evidence of its identity with homovanillic acid was obtained in the following experiment.

An extract of the caudate nucleus of the dog was subjected to one-dimensional chromatography ; the chromatogram was cut into consecutive strips and each strip eluted separately with water. The fluorescence reaction was applied to each eluate. Fig. 5 shows that a fluorescence was developed with material eluted from the region corresponding to the $R_F$ of homovanillic acid. The activation and fluorescence spectra of the fluorescent product are shown in Fig. 6 and are identical with those derived from homovanillic acid. It is therefore concluded that homovanillic acid is normally present in the caudate nucleus of the dog.

The concentrations of homovanillic acid in the caudate nucleus were measured with several species. Extracts were chromatographed in system B and the appropriate region of the chromatogram was eluted and the fluorescence reaction applied to the eluate. The estimated concentrations of homovanillic acid in these tissues are given in Table 2.

TABLE 2

THE CONCENTRATION OF HOMOVANILLIC ACID IN THE CAUDATE NUCLEUS

Values marked with an asterisk refer to pooled tissues

| Species | Homovanillic acid ($\mu$g/g of tissue) |
|---|---|
| Dog | 12·8* |
| | 8·2 |
| | 9·9 |
| | 8·7 |
| Rabbit | 9·1 |
| Cat | 4·0 |
| | 3·0* |
| Sheep | 2·2 |
| | 4·4 |

The recovery of homovanillic acid added to homogenates was generally 60 to 75%, and the figures in Table 2 are corrected on the basis of these recovery values.

## DISCUSSION

The described fluorescence reaction for the estimation of homovanillic acid appears to be a general reaction for a number of compounds related to 4-hydroxy-3-methoxyphenylethane. The lack of absolute specificity requires that a method

of separating such substances be applied to tissue extracts before an estimation of the concentration of any of them can be made.

The nature of the fluorescent substance has not yet been determined, but light-sensitive reactions between organic acids and ferric chloride have been described (Ghosh & Purakayastha, 1929).

The application of the fluorimetric method to the estimation of homovanillic acid in brain tissue extracts has shown that there is normally a high concentration of this acid in the caudate nucleus of animals of several species.

Rosengren (1960) has reported that 3,4-dihydroxyphenylacetic acid is present in the corpus striatum of normal rabbit, pig and man in a concentration of 0.5 $\mu g/g$ or less. The presence of a higher concentration of homovanillic acid in the caudate nucleus indicates the importance of catechol-*O*-methyl transferase as well as that of monoamine oxidase in the metabolism of the endogenous dopamine in the brain.

I wish to thank Roche Products for the 4-hydroxy-3-methoxymandelic acid, Hoffman La Roche, Basle (through the courtesy of Dr A. Pletscher), for the 4-hydroxy-3-methoxyphenylalanine, and Dr J. Axelrod for the 4-hydroxy-3-methoxyphenylglycol. I also wish to thank Dr M. Vogt, F.R.S., for her advice and encouragement during this work.

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# ΗE ESTIMATION OF SMALL QUANTITIES OF DIHYDROXYPHENYLETHYLAMINE IN TISSUES

BY

R. LAVERTY and D. F. SHARMAN

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# THE ESTIMATION OF SMALL QUANTITIES OF 3,4-DIHYDROXYPHENYLETHYLAMINE IN TISSUES

BY

R. LAVERTY* AND D. F. SHARMAN

*From the Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge*

(*Received September* 18, 1964)

Several methods have been used to estimate small amounts of 3,4-dihydroxyphenylethylamine (dopamine) which occur in many plant and animal tissues. The biological assay of dopamine is, by comparison with that of other catechol amines, relatively insensitive. The pressor effect of dopamine on the blood pressure of the rat and the depressor effect observed on the blood pressure of the guinea-pig have been used to assay dopamine extracted from the splenic nerve and stellate ganglion of the ox (Schümann, 1956). These methods are suitable for microgram quantities of dopamine.

Of the chemical methods which are applicable to the estimation of dopamine only those involving the formation of a fluorescent derivative are likely to be sensitive enough to estimate the small amounts of dopamine occurring in some tissues. The main fluorimetric methods are based on two chemical reactions. The first of these was described by Natelson, Lugovoy & Pincus (1949) who showed that catechol amines could condense with ethylenediamine to yield intensely fluorescent compounds. This reaction, which depends on the presence of the catechol group, was developed by Weil-Malherbe & Bone (1957) and used to estimate catechol amines including dopamine. The reaction was also used by Euler & Lishajko (1957) to identify and estimate the dopamine in beef splenic nerve. The major criticism of this method lies in its lack of specificity. The second reaction which has been used involves the conversion of dopamine into an indole derivative by oxidation and treatment with alkali, which then shows a fluorescence in acid solution (Carlsson & Waldeck, 1958). This method has been used with slight modifications by several authors (Drujan, Sourkes, Layne & Murphy, 1959; McGeer & McGeer, 1962) and has the advantage of a greater specificity. In this laboratory the latter method was found to be unworkable, apparently because of the presence of material in the water supply which is not easily removed by distillation. We have therefore been compelled to use the condensation with ethylenediamine as the basis for the estimation of dopamine. This paper describes ways in which the specificity and sensitivity of this method can be improved so that very small quantities of dopamine may be extracted from tissues, identified with reasonable certainty and estimated.

## METHODS

*Materials.* These were ascorbic acid (Roche Products), adrenaline base (Burroughs Wellcome), noradrenaline bitartrate (L. Light), dopamine hydrochloride (California Foundation for Biochemical Research),

* Beit Memorial Research Fellow. Present address: Wellcome Research Institute, University of Otago Medical School, Dunedin, New Zealand.

isoprenaline sulphate (Boots), 6-hydroxydopamine hydrobromide (Merck Sharpe & Dohme), catechol (B.D.H.), α-methyldopamine hydrochloride, and ethylenediamine (May & Baker) distilled at least three times until reagent blank fluorescence was low and constant, dichloromethane (G.P.R.; Hopkin & Williams) distilled once, isobutanol (A.R.) distilled three times, acetic anhydride (A.R.; B.D.H.), sodium chloride (R grade; May & Baker) check reagent blank fluorescence with each new batch distilled three times, and hydrochloric acid (M.A.R.; Hopkin & Williams).

Methanol was purified by distilling from sodium hydroxide and then redistilled. Chromatography solvents were of analytical reagent grade and distilled once before use. All other chemicals were of Analytical Reagent grade.

Other materials were Dowex 50WX-8 cation exchange resin (Dow Chemical Co.), Whatman No. 50 paper for chromatography (extracted with 2 N-sodium hydroxide solution and washed as described by Sharman, 1963), and deionized and distilled water.

The acetylation and extraction of dopamine were based on the methods described by Hagopian, Dorfman & Gut (1961) for the extraction of dopamine and its metabolites from biological media and applied to the estimation of catechol amines in urine by Goldstein, Friedhoff & Simmons (1959).

*The preparation of tissue extracts for acetylation.* Peripheral nervous tissues and spinal cords were frozen in liquid nitrogen and crushed in a stainless steel instrument similar to a tablet die. The powdered tissue was then homogenized in 0.1 N-hydrochloric acid or in 0.1 N-sulphuric acid in a glass homogenizer. Other central nervous tissues were homogenized directly in the acid. In each instance a few mg of ascorbic acid were added to the acid before homogenizing the tissues. If a direct extraction from a deproteinized solution was to be made, sulphuric acid was used and this also served as the protein precipitant. The homogenates prepared in sulphuric acid were left for 15 min at 4° C, centrifuged at 6,000 *g* for 5 min at 0° C and the supernatant fluid was acetylated. In those experiments in which the catechol amines were adsorbed on to a column of Dowex 50WX-8 cation exchange resin, the tissue was homogenized in hydrochloric acid. The proteins were then precipitated by adding perchloric acid to make the solution 0.4 N. Adsorption of the catechol amines onto the resin was carried out essentially as described by Bertler, Carlsson & Rosengren (1958). The total catechol amines were eluted from the column with 8 to 10 ml. of 2 N- or 3 N-hydrochloric acid. Disodium edetate (20 mg) was added to the eluate which was then partially neutralized by the addition of approximately 1.2 g of sodium bicarbonate. The eluates were then ready for acetylation. A reasonable separation of adrenaline and noradrenaline from dopamine could be obtained by eluting the former two amines from the column with 10 ml. of 0.4 N-hydrochloric acid and the latter amine with 8 ml. of 2 N-hydrochloric acid. Two compounds closely related to dopamine, 6-hydroxydopamine and α-methyldopamine, were found to be eluted mainly in the second fraction.

*Acetylation of solutions containing catechol amines.* Acetic anhydride was added to the solution in the proportion of 0.3 ml. of the anhydride for each 4 ml. of solution. A slight excess of powdered sodium bicarbonate was then added in small portions and the solution was shaken as soon as the effervescence subsided. The mixture was ready for extraction when all the acetic anhydride had disappeared. The procedure took about 15 min. Details of the products formed with some catechol amines, usually 3-*O*,4-*O*,*N*-triacetyl derivatives, are described by Welsh (1955).

*Extraction of the acetylated catechol amines.* After acetylation the solution was extracted twice with two times its volume of dichloromethane. The dichloromethane extracts were filtered through anhydrous sodium sulphate, and evaporated to approximately 0.2 ml. under a stream of air in a water bath at 40 to 50° C.

*Paper chromatography.* All chromatograms were developed on alkali-washed Whatman No. 50 paper. This paper shows a minimum of background fluorescence. The sheets of paper were cut into lanes so that each sample was run on a 2-cm-wide strip. Two solvent systems have been used: a slight modification of Bush System C (Bush, 1952) consisting of toluene, ethyl acetate, methanol and water in the proportions 10 : 1 : 5 : 5 by volume. The chromatogram was equilibrated with the aqueous phase for 16 hr and then developed for 4 to 5 hr with the organic phase.

When a separation of 6-hydroxydopamine (a possible metabolite of dopamine; Senoh, Creveling, Udenfriend & Witkop, 1959) from dopamine was required a solvent system based on one of the *tert*.-butanol systems described by Eberlein & Bongiovanni (1955) was found to be suitable. This was a mixture of petroleum spirit (boiling point 80 to 100° C), *tert*.-butanol and water in the proportions 8 : 3 : 8 by volume.

The chromatogram was equilibrated with the aqueous phase for 16 hr and then developed for approximately 18 hr with the organic phase.

The acetylated derivatives of the catechol amines were eluted from small portions of the chromatograms by shaking the piece of paper with 4 ml. of water for 1 hr. The paper was removed before the condensation with ethylenediamine was carried out.

Acetyl derivatives prepared from authentic catechol amines were run on marker strips in parallel with each set of estimations. The marker strips were sprayed with a fivefold dilution of the ethylenediamine–hydrochloric acid mixture described in the following section, placed between two sheets of glass and heated at 80° C for 20 min. The position of the acetyl derivatives of the catechol amines on the chromatogram could be easily seen by the coloured, fluorescing spots which developed. Usually the position of the dopamine derivative was determined and that portion of each chromatogram strip lying between the origin and a point 2 cm in front of the dopamine position was divided into eight or ten equal portions. Each of these was eluted and condensed with ethylenediamine. Measurement of fluorescence yielded a profile of the catechol derivatives present on the chromatogram. In a few experiments the whole length of the chromatogram was divided up to see if other substances giving rise to a fluorescent derivative could be detected.

*Condensation with ethylenediamine.* A mixture of ethylenediamine and 2 N-hydrochloric acid, 3 : 2 by volume, was freshly prepared. Of this solution 0.5 ml. was added to the 4.0 ml. of eluate from the chromatogram and mixed carefully. The mixture was then heated at 60 to 65° C for 20 min in the dark. The samples were removed from the water bath, cooled, saturated with sodium chloride and then shaken with 3.0 ml. of isobutanol for 4 min. After centrifugation, the isobutanol layer was examined fluorimetrically. Standards were prepared by acetylating known amounts of catechol amines in a volume of 4 ml. and condensing them with ethylenediamine. This latter technique of acetylation and condensation without extracting the acetylated derivative can also be applied to the dopamine eluate from resin columns.

*Fluorescence.* Activation and fluorescence spectra were determined in an Aminco-Bowman spectrophotofluorimeter. Wavelengths given in this paper are uncorrected instrumental values. Calibration against a mercury line spectrum indicated an accuracy of $\pm 5$ mμ in the range of the wavelengths of maximum activation and fluorescence. Routine estimations were made on a Locarte filter fluorimeter modified to take square quartz cuvettes ($1.0 \times 1.0 \times 4.4$ cm; internal measurements), and fitted with a device to enable the rapid changing of filters in the fluorescent light path. In a few experiments the volumes used were one-fifth of those described above and the fluorescence was measured in a round cuvette (0.4 cm diameter $\times$ 3.8 cm length; internal measurements). This resulted in a further fourfold increase in the sensitivity of the method.

The primary (activation) filter was a combination of a Corning 3389 filter and a Corning 5113 (half standard thickness) filter with the former filter nearest to the mercury lamp. This set transmits mainly the line of the mercury spectrum at 436 mμ. Two secondary (fluorescence) filters were used so that differential fluorimetry, based on differences in fluorescence spectra, could be carried out. These were Ilford Bright-Spectrum No. 623 (maximum transmission 490 mμ) and Ilford Bright-Spectrum No. 625 (maximum transmission 540 mμ).

The differential fluorimetric estimation of mixtures of dopamine and another catechol amine (called C) was carried out using the following equations. C represents a catechol amine which gives a fluorescence spectrum different from that given by dopamine.

$$\mu\text{g of dopamine} = (BC_1 - AC_2)/(C_1D_2 - C_2D_1)$$

$$\mu\text{g of C} = [A - (\mu\text{g dopamine} \times D_1)]/C_1$$

where $A$ = fluorescence reading with filter 1

$B$ = fluorescence reading with filter 2

$D_1$ = fluorescence for 1.0 μg of dopamine on filter 1

$D_2$ = fluorescence for 1.0 μg of dopamine on filter 2

$C_1$ = fluorescence for 1.0 μg of other catechol amine with filter 1

$C_2$ = fluorescence for 1.0 μg of other catechol amine with filter 2

RESULTS

*The effect of acetylation on the fluorescence obtained by condensing catechol amines with ethylenediamine.* If catechol amines are acetylated before condensing them with ethylenediamine the intensity of the fluorescence developed is increased. Furthermore, the wavelength of maximal fluorescence may be changed. Condensates derived from adrenaline and from 6-hydroxydopamine fluoresce at a shorter wavelength. These changes are shown in Table 1, which also shows that after acetylation dopamine and α-methyldopamine can now be distinguished from those catechol derivatives which before acetylation gave rise to almost the same fluorescence spectrum.

*The separation of the acetyl derivatives of catechol amines on paper chromatograms.* The $R_F$ values of some acetylated catechol amines in the modified Bush system C are given in Table 2. The $R_F$ values obtained for the first three compounds are in agreement with the results of Goldstein, Friedhoff, Simmons & Prochoroff (1959). Table 2 also shows that the derivatives of dopamine and α-methyldopamine are separated in the modified Bush solvent system C. Fig. 1 illustrates the separation of acetylated 6-hydroxydopamine from the dopamine derivative in the *tert.*-butanol solvent system.

*Recovery of catechol amines.* Small quantities of catechol amines were added to tissue homogenates and estimated after acetylation using the direct extraction method described above. Table 3 shows the recovery of noradrenaline and dopamine from tissue homogenates. Dopamine appears to be well recovered from tissue homogenates, but noradrenaline shows a variable and somewhat lower recovery. This difference might be explained by the proximity of the noradrenaline acetate position to the origin of the chromatogram so that other substances might be included in the eluate which cause a reduction in the fluorescence developed from the noradrenaline.

When the resin column stage, which involves a loss of approximately 35%, is included in the extraction procedure the expected overall recovery would be about 40%. In two experiments in which dopamine was recovered from tissue homogenates by this procedure the recovery was 44 and 32%. The estimation of adrenaline has not yet been examined in detail.

*Application to nervous tissue.* In a preliminary survey the methods have been used to make a few or single observations on a wide range of nervous tissues from different species so that the range and applicability of the methods could be examined.

A combination of the resin column extraction, acetylation and paper chromatographic separation of acetylated catechol amines was applied to a selection of nervous tissues obtained from dogs and cats and the dopamine and noradrenaline contents were estimated. The results obtained are given in Table 4. They agree with previous observations on the distribution of dopamine in nervous tissues (Bertler & Rosengren, 1959) and also show the presence of dopamine in sympathetic ganglia other than the stellate ganglion (Schümann, 1956).

Figs. 1 and 2 show the fluorescence profiles of that part of chromatograms of extracts of dog hypothalamus and superior cervical ganglion where the acetylated naturally occurring catechol amines are found. They were developed with the *tert.*-butanol solvent system and show well-defined dopamine peaks. There was, however, a very small amount of fluorescence detected about 18 cm in front of the dopamine region. This has not been

TABLE 1

THE ACTIVATION AND FLUORESCENCE MAXIMA AND THE RELATIVE FLUORESCENCE INTENSITIES OF THE PRODUCTS OBTAINED AFTER CONDENSATION OF CATECHOL AMINES AND THEIR ACETYLATED DERIVATIVES WITH ETHYLENE DIAMINE

The maxima are given for the aqueous solution after condensation and for the isobutanol extract thereof; the relative intensity is for 1 μg of catechol amine in 4 ml. of water and 3 ml. of isobutanol at its maximum wavelengths (uncorrected instrumental values)

| Catechol compound | Direct condensation | | | | | | Condensation after acetylation | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Wavelength maxima in water | | Relative fluorescence intensity | Wavelength maxima in isobutanol | | Relative fluorescence intensity | Wavelength maxima in water | | Relative fluorescence intensity | Wavelength maxima in isobutanol | | Relative fluorescence intensity |
| | Activation (mμ) | Fluorescence (mμ) | | Activation (mμ) | Fluorescence (mμ) | | Activation (mμ) | Fluorescence (mμ) | | Activation (mμ) | Fluorescence (mμ) | |
| Noradrenaline | 410 | 510 | 153 | 420 | 490 | 960 | 410 | 510 | 220 | 420 | 490 | 1,680 |
| Adrenaline | 410 | 530 | 27 | 420 | 525 | 250 | 410 | 510 | 196 | 420 | 490 | 1,510 |
| Dopamine | 410 | 540 | 28 | 420 | 525 | 250 | 410 | 540 | 78 | 430 | 520 | 980 |
| α-Methyldopamine | 410 | 540 | 25 | 420 | 525 | 210 | 410 | 540 | 52 | 430 | 520 | 680 |
| 6-Hydroxydopamine | 410 | 510 | 28 | 420 | 520 | 280 | 410 | 510 | 72 | 415 | 485 | 630 |
| Isoprenaline | 410 | 510 | 16·5 | 420 | 500 | 87 | 410 | 510 | 67 | 420 | 490 | 496 |
| 3,4-Dihydroxyphenylacetic acid | 410 | 530 | 87 | 420 | 490 | 230 | 410 | 530 | 85 | 420 | 490 | 270 |
| Catechol | 410 | 510 | 380 | 420 | 490 | 2,760 | 410 | 510 | 420 | 420 | 490 | 3,000 |

TABLE 2

$R_F$ VALUES OF ACETYLATED CATECHOL DERIVATIVES IN BUSH SOLVENT SYSTEM C

| Acetyl derivative of | $R_F$ |
|---|---|
| Noradrenaline | 0·05 |
| Adrenaline | 0·19 |
| Dopamine | 0·33 |
| 6-Hydroxydopamine | 0·35 |
| α-Methyldopamine | 0·45 |
| Isoprenaline | 0·63 |
| Catechol | 0·90 |

TABLE 3

RECOVERY OF CATECHOL AMINES ADDED TO TISSUE HOMOGENATES

| Amount added (ng) | Amount recovered: Dopamine (ng) | Amount recovered: Noradrenaline (ng) |
|---|---|---|
| 100 | 85 | 58 |
| 100 | 66 | 69 |
| 100 | 77 | 46 |
| 100 | 74 | 58 |
| 100 | 85 | 50 |
| 50 | 37 | 31 |
| 50 | 28 | 17 |
| 25 | 17·5 | 25 |
| 25 | 12 | 6 |

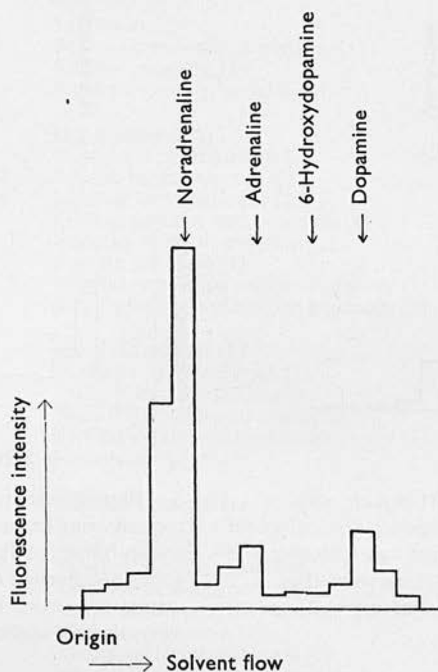


Fig. 1. Fluorescence profile of part of a chromatogram of an extract of dog hypothalamus. Developing solvent—petroleum spirit : *tert*.-butanol : water. Arrows show position of acetyl derivatives of authentic catechol amines.

TABLE 4

THE CONCENTRATION OF NORADRENALINE AND DOPAMINE IN NERVOUS TISSUES

The extraction method included a cation exchange resin column and the results were corrected for a recovery of 40%. Tissues were pooled samples from four cats and three dogs. * These estimations were made on the thoracic and lumbar cord from each of two cats and on a pooled sample of portions of the thoracic cord from two dogs. Concentrations refer to fresh tissue

| | Cat | | | Dog | | |
|---|---|---|---|---|---|---|
| | Weight of tissue | Concentration | | Weight of tissue | Concentration | |
| Tissue | (g) | Noradrenaline (μg/g) | Dopamine (μg/g) | (g) | Noradrenaline (μg/g) | Dopamin (μg/g) |
| Caudate nucleus | 1·71 | 0·10 | 9·9 | 1·64 | 0·09 | 9·9 |
| Hypothalamus | 0·73 | 2·85 | 0·20 | 0·95 | 1·35 | 0·25 |
| Massa intermedia of thalamus | 1·19 | 0·67 | 0·05 | 2·10 | 0·17 | 0·05 |
| Midbrain | 2·22 | 0·58 | 0·15 | 2·54 | 0·43 | 0·33 |
| Cerebral cortex | 1·25 | 0·12 | 0·07 | 1·70 | 0·16 | 0·01 |
| Palaeocerebellum | 1·21 | 0·12 | <0.003 | 1·61 | 0·05 | 0·003 |
| Spinal cord* | 1·91 | 0·16 | 0·007 | 1·00 | 0·10 | 0·008 |
| | 2·89 | 0·08 | 0·010 | | | |
| Splenic nerve | | — | — | 0·26 | 12·2 | 0·45 |
| Cardiac nerve | | — | — | 0·07 | 7·8 | 0·78 |
| Sympathetic chain | | — | — | 0·65 | 2·2 | 0·17 |
| Superior cervical ganglion | | — | — | 0·35 | 26·5 | 2·00 |
| Inferior cervical ganglion | | — | — | 0·20 | 5·5 | 0·80 |
| Stellate ganglion | | — | — | 0·58 | 15·9 | 1·53 |

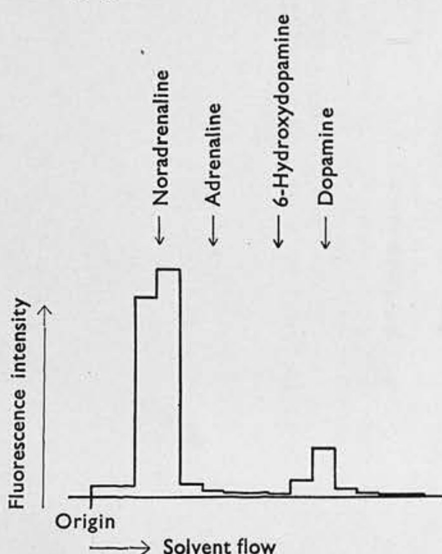


Fig. 2. Fluorescence profile of part of a chromatogram of an extract of dog superior cervical ganglion. Developing solvent—petroleum spirit : *tert*.-butanol : water. Arrows show the position of acetyl derivatives of authentic catechol amines.

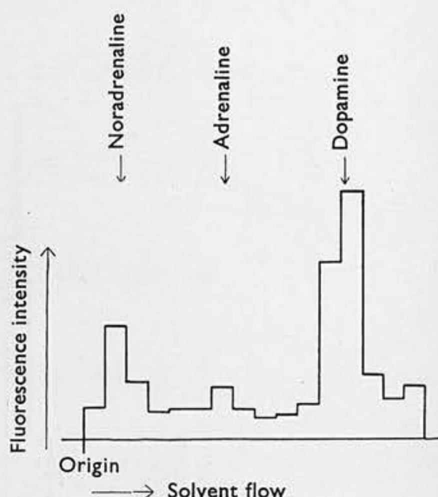


Fig. 3. Fluorescence profile of part of a chromatogram of an extract of the median eminence and pituitary stalk of the cat. Developing solvent—toluene : ethyl acetate : methanol : water. Arrows show position of acetyl derivatives of authentic catechol amines.

identified. The results obtained from experiments in which the acetylated catechol amines were extracted directly from deproteinized solutions are given in Table 5. They demonstrate the presence of dopamine in the superior cervical ganglion of several species and also show

that dopamine is a major catechol amine in the pituitary stalk and median eminence, a result which has been suggested by the histological and pharmacological examination of this tissue by Fuxe (1964).

The estimation of the dopamine in a single pituitary stalk and median eminence of the cat is at the lower limit of the sensitivity of the method. By using the tissue from two cats and employing the microcell it is possible to find not only dopamine, but also adrenaline and noradrenaline in the pituitary stalk and median eminence of the cat. A profile of the fluorescence from such a chromatogram is given in Fig. 3 and shows the presence of the three catechol amines in this tissue.

TABLE 5

THE CONCENTRATION OF NORADRENALINE AND DOPAMINE IN NERVOUS TISSUES

Results were obtained by extracting the acetylated amines directly from deproteinized tissue homogenates. Number of animals from which pooled tissue sample was obtained are given in parentheses. Concentrations are in μg/g of fresh tissue corrected for a mean recovery of 71% for dopamine and 56% for noradrenaline. * Not estimated

| Species | Weight (mg) | Tissue | Noradrenaline | Dopamine |
|---|---|---|---|---|
| Rat | 132 | Caudate nucleus (3) | 0·27 | 6·39 |
| | 116 | Hypothalamus (3) | 1·29 | 0·14 |
| | 199 | Midbrain (3) | 0·55 | 0·13 |
| | 165 | Cerebral cortex (3) | 0·18 | <0.01 |
| Rabbit | 87 | Hypothalamus (1) | 1·52 | 0·20 |
| | 610 | Midbrain (2) | 0·59 | 0·18 |
| | 88 | Massa intermedia of thalamus (1) | 0·36 | 0·10 |
| | 10 | Stellate ganglion (1) | 13·0 | 4·64 |
| | 25 | Superior cervical ganglion (1) | 3·5 | 0·85 |
| Cat | 69 | Hypothalamus (1) | 2·43 | 0·20 |
| | 78 | Superior hypothalamus (2) | 1·23 | 0·35 |
| | 108 | Medial hypothalamus (2) | 2·59 | 0·25 |
| | 52 | Inferior hypothalamus (2) | 2·89 | 0·81 |
| | 102 | Globus pallidus and putamen (1) | 0·05 | 8·22 |
| | 34 | Superior cervical ganglion (1) | 5·25 | 0·82 |
| | 44 | Stellate ganglion (1) | 2·82 | 1·32 |
| | 7 | Pituitary stalk and median eminence (1) | 1·1 | 1·3 |
| | 20 | Pituitary stalk and median eminence (2) | 2·9 | 8·7 |
| Sheep | 81 | Caudate nucleus (1) | * | 11·78 |
| | 178 | Superior hypothalamus (1) | 3·01 | 0·27 |
| | 218 | Inferior hypothalamus (1) | 1·44 | 0·21 |
| | 256 | Massa intermedia of thalamus (1) | 0·70 | 0·34 |
| | 36 | Pituitary stalk and median eminence (1) | 0·32 | 5·05 |
| | 400 | Stellate ganglion (1) | 4·07 | 1·48 |
| Goat | 156 | Caudate nucleus (1) | * | 10·9 |
| | 253 | Superior hypothalamus (1) | 1·48 | 0·15 |
| | 222 | Medial hypothalamus (1) | 1·81 | 0·10 |
| | 85 | Inferior hypothalamus (1) | 2·16 | 0·11 |
| | 244 | Massa intermedia of thalamus, medial (1) | 0·34 | 0·31 |
| | 283 | Massa intermedia of thalamus, lateral (1) | 0·12 | 0·46 |
| | 445 | Anterior midbrain (1) | 0·29 | 0·41 |
| | 342 | Medial anterior midbrain (1) | 0·45 | 0·27 |
| | 302 | Medial posterior midbrain (1) | 0·55 | 0·14 |
| | 425 | Posterior midbrain (1) | 0·34 | 0·04 |
| | 39 | Pituitary stalk and median eminence (1) | 0·16 | 2·0 |
| | 165 | Stellate ganglion (1) | 1·95 | 0·85 |

## DISCUSSION

The method described here for the detection, identification and estimation of small quantities of dopamine has overcome some of the difficulties of the earlier methods in which ethylenediamine was used to estimate catechol amines. It is possible to identify as little as 10 to 20 ng of dopamine in tissue. The specificity of the fluorescence developed with dopamine has been improved by acetylating the catechol amines before condensing them with ethylenediamine and by determining the position of the acetates on a paper chromatogram. Of the catechol amines normally present in tissues only dopamine will give rise to a product having a maximum fluorescence at 520 m$\mu$. $\alpha$-Methyldopamine was the only other catechol compound of those investigated which gave rise to a similar fluorescence. The acetyl derivative of this substance was separated from the dopamine triacetate by paper chromatography and thus, if present, would not interfere with the dopamine estimations. 3,4-Dihydroxyphenylalanine (dopa) and 3,4-dihydroxyphenylacetic acid were not extracted by the procedures used for the catechol amines. A small amount of fluorescence was obtained when 3,4-dihydroxymandelic acid was acetylated, extracted with dichloromethane and then condensed with ethylenediamine. This might be due to the presence of 3,4-dihydroxybenzaldehyde, formed by oxidation, in the sample of acid used. 3,4-Dihydroxybenzaldehyde, 3,4-dihydroxyphenylethanol and 3,4-dihydroxyphenylglycol, possible catechol intermediates in the metabolism of catechol amines, have not yet been examined, but they would not be included in the eluates from a cation exchange resin column. Under normal conditions these latter substances might be expected to be present in tissues in very small quantities only, but must be considered when processes altering the metabolism of catechol amines are being investigated.

One disadvantage of the present method when the resin column stage is omitted, is its inability to distinguish between dopamine and *N*-acetyldopamine, a substance which can be formed *in vivo* (Goldstein & Musacchio, 1962) and also between other catechol amines and their *N*-acetyl derivatives. However, the results of Goldstein (1964) appear to indicate that *N*-acetyl derivatives of dopamine and its metabolites do not occur in the brain tissue of the rat after administration of dopa. Thus it can be concluded that the method can be used to identify and estimate small quantities of dopamine with reasonable certainty and accuracy. The estimation of noradrenaline by the method as it stands is not as good as that of dopamine. This is probably explained by the proximity of the noradrenaline acetate region to the origin of the chromatogram and resultant interference with the development of the fluorescence by material left at the origin. This could probably be overcome by developing the chromatogram for a longer time. A further difficulty lies in the estimation of noradrenaline in the presence of a large amount of dopamine. The acetylation of dopamine resulted in the formation of about 99.5% of the 3,*O*,4-*O*,*N*-triacetyl derivative and there was about 0.5% of an incompletely acetylated derivative present on the chromatogram which runs to the same place as the noradrenaline triacetate. The two substances can be estimated by differential fluorimetry but a small amount of noradrenaline in the presence of a large amount of dopamine can be easily overlooked. This fact also limits the use of the method for radioactive estimations.

The methods described here are applicable to the estimation of small quantities of dopamine in tissues. When larger amounts of tissues (0.5 to 5.0 g) are involved it is advisable to use the resin column stage in the extraction, although the recovery of dopamine is rather

low. For quantities of tissues below 0.5 g this stage can be omitted with a concomitant increase in the recovery of dopamine, but the observations made above with regard to other catechol derivatives must be taken into account.

The amount of dopamine estimated in various parts of the nervous system is, in general, in agreement with that found by other workers (Bertler & Rosengren, 1959). However, only very small quantities of dopamine have been detected in spinal cord, a result which differs from the observations of McGeer & McGeer (1962) who reported a concentration of 0.45 $\mu$g/g in the spinal cord of the cat. Observations made in this laboratory have shown that the concentration of dopamine in tissues can be extremely variable and it is certainly advisable to examine the spinal cord more fully. According to Hamberger, Norberg & Sjöqvist (1964), Holmstedt and his colleagues have been unable to detect dopamine in significant amounts in the superior cervical ganglion of the cat. The present experiments clearly demonstrate the presence of this amine in all the sympathetic ganglia which were examined and indicate the degree of sensitivity of the method. This is further illustrated by the demonstration that the major catechol amine in the median eminence and pituitary stalk of the cat, sheep and goat is dopamine.

## SUMMARY

1. Because of difficulties encountered in the estimation of small amounts of dopamine, a method has been developed that enables the identification and estimation of as little as 10 to 20 ng of this substance.

2. Dopamine and $\alpha$-methyldopamine can be distinguished from a number of other catechol derivatives by first acetylating these substances with acetic anhydride in the presence of sodium bicarbonate and then condensing the acetyl derivatives with ethylenediamine. The condensates formed from dopamine and $\alpha$-methyldopamine fluoresce at a longer wavelength than the condensates derived from the other compounds. The intensity of the fluorescence developed is also increased by acetylating the catechol amines before condensing them with ethylenediamine.

3. Acetylated catechol amines are easily extracted from aqueous solutions into dichloromethane.

4. The acetyl derivatives of catechol amines can be separated by paper chromatography. Two solvent systems used were toluene, ethyl acetate, methanol and water (10 : 1 : 5 : 5), and petroleum spirit, *tert*.-butanol and water (8 : 3 : 8). The former system separated acetylated dopamine from acetylated $\alpha$-methyldopamine. The latter system was required to separate acetylated dopamine from acetylated 6-hydroxydopamine.

5. The catechol amines in extracts prepared from nervous tissues were acetylated, extracted and then separated by paper chromatography. They were then estimated by differential fluorimetry after condensing the acetyl derivatives with ethylenediamine. Dopamine was present in the superior cervical ganglia of the dog, the cat and the rabbit. The major catechol amine in the pituitary stalk and median eminence of the cat, the goat and the sheep was dopamine.

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# ODIFICATION BY DRUGS OF THE METABOLISM OF 3,4-DIHYDROXYPHENYLETHYLAMINE, ORADRENALINE AND 5-HYDROXYTRYPTAMINE IN THE BRAIN

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**R. LAVERTY and D. F. SHARMAN**

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# MODIFICATION BY DRUGS OF THE METABOLISM OF 3,4-DIHYDROXYPHENYLETHYLAMINE, NORADRENALINE AND 5-HYDROXYTRYPTAMINE IN THE BRAIN

BY

R. LAVERTY* AND D. F. SHARMAN

*From the Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge*

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There have been a number of studies of the effects of drugs on the content of various amines found in brain tissue. In particular, the concentrations in the brain of noradrenaline (Vogt, 1954) and 5-hydroxytryptamine (Paasonen & Vogt, 1956; Brodie, Shore & Pletscher, 1956) have been determined before and after treatment with drugs, and many attempts have been made to correlate the change in brain amine concentration with an observable change in animal behaviour. Later, brain dopamine (3,4-dihydroxyphenylethylamine) has been included in such studies (Bertler, 1961).

In the present experiments, the content of dopamine, noradrenaline and 5-hydroxytryptamine in different parts of cat and dog brain was measured, and so was the content of homovanillic acid (4-hydroxy-3-methoxyphenylacetic acid) and 5-hydroxyindol-3-ylacetic acid, the major acid metabolites of dopamine and 5-hydroxytryptamine. The corresponding acid metabolite of noradrenaline, vanillylmandelic acid (4-hydroxy-3-methoxymandelic acid), has not been detected in brain tissue (Andén, Roos & Werdinius, 1964; Sharman, unpublished) and so could not be determined. The simultaneous determination of an amine and its metabolite permitted a study of the effect of the drug not only on the content of the amine but also on a possible indicator of the rate of metabolism of the amine.

The concentrations of both dopamine and homovanillic acid are highest in the brain in the caudate nucleus and putamen; since the caudate nucleus is easy to dissect out reproducibly it was used for the estimation of these two compounds. The caudate nucleus is thought to form part, with other basal ganglia which also contain dopamine, of the " extrapyramidal motor system", though recent evidence suggests that it has a more generalized function, acting as an integrative centre for the whole of the cortex (Laursen, 1963; Carman, Cowan & Powell, 1963). In the brains from patients with Parkinson's syndrome, Bernheimer, Birkmayer & Hornykiewicz (1963) found a much-reduced concentration of dopamine in the caudate nucleus, as well as smaller reductions in the concentrations in the brain of noradrenaline and 5-hydroxytryptamine. This suggests a relationship between brain dopamine metabolism and Parkinson's syndrome, as do the results of Barbeau &

* Beit Memorial Research Fellow. Present address: Wellcome Research Institute, University of Otago Medical School, Dunedin, New Zealand.

Sourkes (1961) who found a reduced urinary excretion of dopamine in patients with Parkinsonism. For this reason we have studied a number of drugs which are reported to influence or induce a Parkinson-like syndrome in animals or man. In addition, some drugs were studied which cause or mimic central sympathetic excitation. Preliminary reports of some of the results have been published (Laverty, 1963; Sharman, 1963b).

## METHODS

Adult cats, dogs and rabbits were used. Whenever possible litter mates were used as controls for the treated animals. If litter mates were not available, the experimental and control groups were usually matched for sex and size. The brains from control animals were analysed simultaneously with those from treated animals; in most experiments, control and treated animals were housed under identical conditions before and during treatment.

Drugs were administered subcutaneously, except for some cats where intraperitoneal or oral administration was used and a few rabbits to which the drugs were given either intraperitoneally or intravenously into a marginal ear vein.

The animals were killed by bleeding during chloroform anaesthesia. The brain was removed and the caudate nuclei and the hypothalamus were removed and deep-frozen; in cats the thalamus (excluding the medial geniculate bodies) was also removed and frozen. All tissues were weighed frozen. Within 2 hr of removal of the tissue from the animal, the hypothalamus and caudate nuclei were each homogenized with at least twice their weight of 0.1 N-hydrochloric acid, with the addition of a few mg of ascorbic acid. The protein in the hypothalamic homogenate and in a portion of the caudate nucleus homogenate was precipitated with an equal volume of 0.8 N-perchloric acid and centrifuged off at 0° C. The *p*H of the supernatant fluid was adjusted to *p*H 4 with 3 N-potassium carbonate; the precipitated potassium perchlorate was removed by further centrifugation at 0° C. The supernatant fluid was then applied to a Dowex 50 X-8 ion-exchange resin column (25 × 4 mm) prepared as described by Bertler, Carlsson & Rosengren (1958). The column was fitted with a capillary tube which maintained a flow rate of 8 to 10 ml./hr without applied pressure. When the supernatant fluid had run through, the column was washed with 4 ml. of water; then the noradrenaline was eluted with 8 ml. of 0.4 N-hydrochloric acid. A further fraction which contained dopamine could be obtained by eluting with 8 ml. of 2 N-hydrochloric acid. The columns used for caudate nucleus extracts were washed with 6 ml. of 0.4 N-hydrochloric acid instead of water to remove any noradrenaline or dihydroxyphenylalanine, and the dopamine was eluted with 8 ml. of 2 N-hydrochloric acid. Edetic acid disodium salt (20 $\mu$g) was added to the dopamine eluates which were then brought to *p*H 4 with solid sodium bicarbonate.

Noradrenaline in the eluates was determined fluorimetrically by a ferricyanide oxidation method (Sharman, Vanov & Vogt, 1962). The fluorescence was measured using a Locarte filter fluorimeter with a Chance OX1 primary filter, and an Ilford 625 secondary filter. This filter combination gave equivalent fluorescence with equal amounts of noradrenaline and adrenaline; the results given for noradrenaline include any adrenaline present. In many experiments the adrenaline content of the noradrenaline eluate was also measured by a differential method using a second filter set; the primary was a combination of Corning 3389 and Corning 5113 and the secondary, Chance OY4. With this set, adrenaline gave approximately three times the fluorescence given by an equal amount of noradrenaline.

Dopamine was measured fluorimetrically by a modification of the method of Weil-Malherbe & Bone (1952). To 4 ml. of neutralized column eluate was added 0.3 ml. of ethylenediamine and 0.2 ml. of 2 N-hydrochloric acid (instead of 0.2 ml. of 2 N-ethylenediamine dihydrochloride) and the mixture was heated in the dark for 20 min at 65° C. The reaction mixture was saturated with sodium chloride and extracted with 3 ml. of isobutanol. The fluorescence of the isobutanol extract was read in an Aminco-Bowman spectrophotofluorimeter at 415/520 m$\mu$ (uncorrected instrumental wavelengths). In some later experiments the dopamine in the eluate was acetylated before condensation (Laverty & Sharman, 1965); this increased the sensitivity of the method.

5-Hydroxytryptamine and 5-hydroxyindol-3-ylacetic acid were measured fluorimetrically as described by Ashcroft & Sharman (1962); in dogs a portion of the caudate nucleus homogenate and in cats the

thalamus was used. Homovanillic acid was measured in portions of the homogenates from cat and dog caudate nuclei by the method described by Sharman (1963a). In rabbits only catechol amines were estimated.

In experiments with tritiated dopamine, the radioactivity was measured using a Packard Tri-Carb liquid scintillation counter. Samples of aqueous solution, usually 1 ml., were added to 15 ml. of scintillator solution in dioxane (Bray, 1960) and counted directly with an efficiency of 10 to 13% against an internal standard of tritiated dopamine. The 1-[$^3$H]-dopamine was obtained from New England Nuclear Corp. (150 $\mu$C/mg).

The following drugs were used: atropine sulphate (B.D.H.), atropine methylbromide, caramiphen hydrochloride (Geigy), chlorpromazine hydrochloride (May & Baker), dexamphetamine sulphate, 3,4-dihydroxyphenylalanine (dopa, L. Light), diethazine hydrochloride (May & Baker), ephedrine hydrochloride, guanethidine (Ciba), morphine hydrochloride (B.D.H.), oxotremorine (May & Baker), pentobarbitone sodium (Abbott), reserpine (Ciba), $\beta$-tetrahydronaphthylamine hydrochloride (Theo. Schuchardt, Munich), trifluoperazine dihydrochloride (Smith, Kline & French), thioproperazine dimethanesulphonate (May & Baker) and thioridazine hydrochloride (Sandoz). Doses are given in terms of the salts.

Standard solutions of amines and metabolites were prepared, in terms of free base or acid, from adrenaline base (Burroughs Wellcome), dopamine hydrochloride (California Corp. for Biochemical Research), homovanillic acid (California Corp. for Biochemical Research), 5-hydroxytryptamine hydrogen oxalate (Regis), 5-hydroxyindol-3-ylacetic acid (L. Light) and noradrenaline bitartrate (L. Light).

## RESULTS

*Recovery experiments.* A number of experiments were carried out on the recovery of dopamine and noradrenaline added to various tissue samples. The recovery from 0.2 $\mu$g of noradrenaline added to cat, dog and rabbit brain tissue samples was 78$\pm$2.6% (mean and standard error) in twenty-eight determinations and was reasonably consistent and reproducible. Dopamine recoveries tended to be more variable; the mean recovery from 0.5 $\mu$g of dopamine added to cat, dog and rabbit tissue in fifty-five estimations was 64$\pm$3.3%.

The recovery figures were calculated from the difference measured between two samples of tissue homogenate, one of which contained added amine; each was run through the entire column extraction procedure. In an attempt to reduce the variability arising from the use of two separate columns, radioactive dopamine was added to a tissue homogenate and the recovered radioactivity measured. This should enable the estimation of the

TABLE 1

COMPARISON OF THE RECOVERIES OF ADDED DOPAMINE (0·5 $\mu$G) ESTIMATED BY FLUORIMETRIC AND RADIOACTIVE TRACER TECHNIQUES

Values are means and standard errors

| Tissue | No. of experiments | Recovery (%) Fluorimetric | Radioactive |
|---|---|---|---|
| Cat caudate nucleus | 6 | 74$\pm$ 7·9 | 83$\pm$1·1 |
| Cat cortex | 5 | 67$\pm$ 1·3 | 82$\pm$2·1 |
| Rabbit caudate nucleus | 6 | 79$\pm$10·2 | 72$\pm$6·2 |
| Rabbit cortex | 10 | 53$\pm$ 7·5 | 62$\pm$1·7 |

unknown dopamine content and of the recovery in the same sample. A number of control experiments (Table 1) were done in which the recovery estimated by fluorimetric means was compared with the recovery estimated by radioactivity. It will be seen that the two recoveries were similar but that the recovery estimated by fluorimetric means was much more variable. This may reflect losses in some experiments of the dopamine in the final

eluate without a corresponding loss of radioactivity, or may reflect a larger experimental error involved in fluorimetric estimations.

Radioactive dopamine was used to detect where the losses occurred in the extraction procedure. When radioactive dopamine was added to a homogenate of mouse lung and the dopamine losses followed at various stages it was found, in four experiments, that approximately 5% was trapped in the precipitated protein and 2% in the potassium perchlorate precipitate; a further 3% was not adsorbed on the resin and the main loss (12%) occurred when the resin was washed with 0.4 N-hydrochloric acid. The remaining radioactivity (78%) was recovered in the 2 N-hydrochloric acid eluate.

Radioactive tracer methods also gave information about the efficiency of the ethylenediamine reaction and isobutanol extraction. It was found that 39% of the radioactivity in the eluate was recovered in the isobutanol extract used to measure the fluorescence developed. The exact reaction products are unknown (Harley-Mason & Laird, 1959) but it is apparent that the reaction between dopamine and ethylenediamine to form an isobutanol-soluble fluorescent product was being operated at less than maximum yield.

Recoveries of 5-hydroxytryptamine (0.2 $\mu$g) and 5-hydroxyindol-3-ylacetic acid (0.2 $\mu$g) added to tissue samples averaged $69 \pm 1.4\%$ (eleven experiments) and $51 \pm 2.0\%$ (eleven experiments) respectively. The mean recovery of homovanillic acid (2 to 5 $\mu$g) added to dog tissue was $72 \pm 6.2\%$ (eleven experiments).

The results presented in this paper are uncorrected for losses.

The amounts of adrenaline and dopamine in the hypothalamus of the animals used in this study were measured, but the results have not been given in detail, as the amounts present were too close to the lower limits of sensitivity of the methods for great reliance to be placed upon them. Single doses of morphine, $\beta$-tetrahydronaphthylamine, dexamphetamine, ephedrine, pentobarbitone or atropine, and chronic treatment with phenothiazines did not change the ratio of adrenaline to noradrenaline in the cat hypothalamus. The adrenaline found in the hypothalamus as a percentage of the total adrenaline plus noradrenaline was 4% in cats and 11% in dogs. This agreed with previous results (Vogt, 1954).

The determination by the ethylenediamine reaction of small amounts of dopamine found in the hypothalamus yielded values of about 0.5 $\mu$g/g but cannot be relied upon as tissue blank figures were high relative to the amount of dopamine present. By using a more sensitive method involving acetylation, separation of the acetylated amine and measurement at two different fluorescence wavelengths (Laverty & Sharman, 1965) it is possible to show that the dopamine content of cat and dog hypothalamus is approximately 0.2 $\mu$g/g.

A striking feature of the present experiments is the extreme variability of the dopamine content of the caudate nucleus (range 2.1 to 12.3 $\mu$g/g in the cat). This is not due to excessive variability in the extraction methods, since in fifteen experiments in which duplicate estimations were made on each sample there was agreement between the duplicate samples and the variation between animals was still present. The variability of the dopamine content appeared to be as great or greater between different groups of animals as within groups, particularly with the cats, and was probably due to poor control over their previous environment. In dogs and rabbits the use of litter mates reduced but did not eliminate the variability. A wide range (0.12 to 0.49 $\mu$g/g) was also observed for the concentration of 5-hydroxytryptamine in the thalamus of the cat.

*Excitant drugs*

*Biochemical effects.* The effect on the concentration of amines and metabolites in the brain of a group of drugs which cause forms of excitation involving sympathetic mechanisms in cats was studied in the cat, dog and rabbit. The results of these experiments are summarized in Table 2.

*Cats.* In confirmation of previous work (Vogt, 1954), it was found that the noradrenaline content of the cat hypothalamus was low 4 hr after injection of morphine and β-tetrahydronaphthylamine, but normal after ephedrine. In the caudate nucleus, the homovanillic acid content was raised 4 hr after the first two, but not after the third drug. Dexamphetamine only slightly lowered the hypothalamic noradrenaline and slightly raised the homovanillic acid level. Yet dexamphetamine was the only drug of these four to lower significantly the dopamine content of the caudate nucleus. In whole rat brain dexamphetamine was seen to lower the noradrenaline content without affecting that of dopamine (Baird & Lewis, 1963).

The peculiar " rage-like " effect seen in cats after the injection of tremorine or its metabolite, oxotremorine (Toman, 1963), prompted a study of the effects of oxotremorine. The drug was given in two doses (0.25 and 0.125 mg/kg, intravenously) spaced at an interval of 2 hr in cats protected from the peripheral effects of the drug by an injection of atropine methylbromide (1.5 mg/kg, subcutaneously) 15 min before the first injection. The only biochemical change found after treatment with oxotremorine was a rise in the homovanillic acid content of the caudate nucleus. Small rises in the 5-hydroxytryptamine concentration were obtained with morphine and β-tetrahydronaphthylamine and a small fall with dexamphetamine; in addition, morphine produced a small increase in the concentration of 5-hydroxyindol-3-ylacetic acid.

*Dogs.* Only β-tetrahydronaphthylamine and dexamphetamine were tested. Both lowered hypothalamic noradrenaline content but, in contrast to the observation on cats, the homovanillic acid level did not rise, nor did that of dopamine fall after amphetamine, indicating a greater susceptibility of the dopamine metabolism in cats than in dogs.

*Rabbits.* Morphine, β-tetrahydronaphthylamine and dexamphetamine had no effect on the dopamine content of the caudate nucleus of rabbits. Though, with the exception of morphine, these drugs had pronounced excitatory effects, only a slight fall in hypothalamic noradrenaline level was observed after amphetamine.

*Behavioural effects.* Morphine in cats caused an obvious central excitation which ranged from "hallucinations " to an increase in motor activity, hissing and occasional convulsions. There was also increased salivation, mydriasis and panting. Rabbits were, if anything, sedated by morphine and respiration was depressed.

β-Tetrahydronaphthylamine caused in cats, dogs and rabbits pupillary dilatation, salivation and intense excitement characterized by increased alertness, apprehension and bouts of violent activity. From time to time, some cats and rabbits showed muscular rigidity and tremor. There was usually panting and an increase in body temperature.

Dexamphetamine caused alertness in all species, but differed from β-tetrahydronaphthylamine in causing specific patterns of motor activity. Cats showed rhythmic head movements and paced about the cage, dogs continually circled the pen and rabbits chewed the bars of the cage. These activities were accompanied by a lack of response to external stimuli but

TABLE 2

THE EFFECT OF SOME EXCITANT DRUGS ON THE MEAN CONTENTS OF SOME AMINES AND THEIR ACID METABOLITES IN BRAIN TISSUE

The noradrenaline content was measured in the hypothalamus, dopamine and homovanillic acid in the caudate nucleus and the indole derivatives in the thalamus of cats and the caudate nucleus of dogs. Values are means and standard errors with numbers of observations in parentheses; one determination of each compound was made in each animal. With groups of less than three animals, actual values are given. * $P<0·05$ and ** $P<0·01$, significant difference from own control animals; † $P<0·05$ and ‡ $P<0·01$, significant difference from all control animals. I.p., Intraperitoneal; s.c., subcutaneous; i.v., intravenous

| Species | Drug | Dose (mg/kg) and duration of treatment | Mean tissue content (μg/g fresh tissue) of | | | | |
|---|---|---|---|---|---|---|---|
| | | | Noradrenaline | Dopamine | Homovanillic acid | 5-Hydroxy-tryptamine | 5-Hydroxyindol-3-yl-acetic acid |
| Cat | Morphine | 50 i.p., 1 hr | 1·6±0·25 (3) | 8·3±0·6 (3) | — | — | — |
| | Morphine | 30 i.p., 4 hr | 0·9±0·17 (4)‡ | 10·0±1·8 (4) | — | — | — |
| | Control | — | 1·4±0·19 (4) | 10·0±1·5 (4) | — | — | — |
| | Morphine | 30 s.c., 4 hr | 1·0±0·12 (8)‡** | 9·2±0·5 (9) | 4·1±0·8 (9)‡ | 0·38±0·03 (9)‡ | 0·34±0·03 (9)‡* |
| | Control | — | 2·0±0·25 (4) | 8·0±1·1 (4) | 2·1±0·3 (4) | 0·29±0·04 (4) | 0·23±0·02 (4) |
| | β-Tetrahydro-naphthylamine | 30 s.c., 4 hr | 0·9±0·08 (7)‡** | 10·2±1·2 (7) | 6·8±0·9 (7)‡* | 0·47±0·03 (5)‡* | 0·23±0·04 (5) |
| | Control | — | 1·7±0·15 (3) | 11·3±0·9 (3) | 2·5±1·0 (3) | 0·36±0·02 (3) | 0·24±0·02 (3) |
| | Dexamphetamine | 10 s.c., 4 hr | 1·6±0·15 (12)‡** | 4·0±0·4 (13)‡** | 2·7±0·3 (6)†* | 0·22±0·02 (13)‡* | 0·22±0·02 (6) |
| | Control | — | 2·0±0·10 (14) | 7·1±0·7 (14) | 2·1±0·1 (9) | 0·29±0·02 (14) | 0·21±0·01 (9) |
| | Ephedrine | 50 s.c., 4 hr | 2·5, 2·2 | 5·4, 6·9 | 1·6, 1·9 | 0·35, 0·39 | 0·22, 0·23 |
| | Control | — | 2·2 | 6·2 | 1·6 | 0·37 | 0·23 |
| | Oxotremorine | 0·375 i.v., 3 hr | 1·7±0·27 (4) | 7·0±0·3 (4) | 4·1±0·2 (4)‡** | 0·23±0·03 (4) | 0·25±0·01 (3) |
| | Control | — | 2·0±0·22 (4) | 7·2±0·4 (4) | 1·7±0·2 (4) | 0·20±0·02 (4)† | 0·23±0·01 (4) |
| | Total controls | — | 1·9±0·09 (30) | 8·0±0·5 (30) | 2·0±0·2 (21) | 0·29±0·02 (26) | 0·22±0·01 (21) |

TABLE 2—(*continued*)

| Species | Drug | Dose (mg/kg) and duration of treatment | Mean tissue content (μg/g fresh tissue) of: Noradrenaline | Dopamine | Homovanillic acid | 5-Hydroxy-tryptamine | 5-Hydroxyindol-3-yl-acetic acid |
|---|---|---|---|---|---|---|---|
| Dog | β-Tetrahydro-naphthylamine | 30 s.c., 4 hr | 0·4±0·12 (3)‡* | 7·7±0·3 (3) | 10·0±1·1 (3) | 0·23±0·05 (3) | 0·14±0·04 (3) |
| | Control | — | 1·3, 1·1 | 7·1, 7·1 | 13·3, 13·5 | 0·23, 0·24 | 0·08, 0·19 |
| | Dexamphetamine | 10 s.c., 4 hr | 0·9±0·12 (4)‡* | 5·7±0·4 (4) | 9·9±0·2 (4)† | 0·15±0·02 (4)‡* | 0·17±0·03 (4) |
| | Control | — | 1·6, 1·7 | 5·8, 6·0 | 10·8, 17·4 | 0·26, 0·31 | 0·19, 0·20 |
| | Total controls | — | 1·6±0·09 (4) | 6·5±0·3 (4) | 13·8±1·4 (4) | 0·26±0·02 (4) | 0·17±0·03 (4) |
| Rabbit | Morphine | 15–80 i.v., 1 hr | 1·1±0·20 (5)† | 9·1±1·1 (5) | — | — | — |
| | Control | — | 1·6±0·25 (5) | 9·9±0·8 (5) | — | — | — |
| | Morphine | 50 i.p., 4 hr | 1·7±0·16 (5) | 8·9±0·8 (4) | — | — | — |
| | Control | — | 1·5±0·14 (6) | 6·9±1·4 (6) | — | — | — |
| | β-Tetrahydro-naphthylamine | 15 i.v., 4 hr | 1·5±0·17 (4) | 10·3±0·7 (4) | — | — | — |
| | Control | — | 1·7±0·12 (4) | 8·3±0·7 (4) | — | — | — |
| | Dexamphetamine | 15 s.c., 4 hr | 1·0±0·06 (3)†* | 5·9±1·1 (3) | — | — | — |
| | Control | — | 1·5±0·15 (4) | 4·5±0·2 (4)† | — | — | — |
| | Total controls | — | 1·5±0·09 (17) | 7·8±0·6 (16) | — | — | — |

occasionally also by aggressiveness. There was salivation, mydriasis and panting, and an increased body temperature. Ephedrine was used in two cats as a control for amphetamine; they became alert and irritable but did not show the specific patterns of motor activity seen after amphetamine.

Oxotremorine caused an immediate response consisting of extreme " rage " and aggression, which was, however, quite different from that produced by other drugs. The animal's actions were much more co-ordinated, closely resembling the actions of a normal cat playing with its captured prey; there was purposeful response to external stimuli such as an immediate attack upon another cat placed in the same cage. The effect lasted approximately 1.5 hr; a subsequent dose (0.125 mg/kg, intravenously) given 2 hr after the first caused a lesser and briefer response of a similar pattern.

*Tranquillizing phenothiazines*

*Biochemical effects.* Four phenothiazine derivatives used as tranquillizers in patients were examined in cats for their effect on the content of amines and their metabolites in brain tissue. Table 3 summarizes the results. Chlorpromazine and thioridazine were compared 4 hr after a single administration. Chlorpromazine (10 mg/kg) lowered the dopamine content and increased the homovanillic acid content of the caudate nucleus but did not lower the hypothalamic noradrenaline level. In contrast, single doses of thioridazine (10 and 50 mg/kg) decreased the concentration of noradrenaline but did not lower the dopamine level. Like chlorpromazine, they increased the homovanillic acid content of the caudate nucleus. The 5-hydroxytryptamine content was lowered by the larger dose. After chronic administration of chlorpromazine, trifluoperazine and thioproperazine the only change observed was an increase in the content of homovanillic acid in the caudate nucleus, whereas thioridazine was without effect on any of the compounds measured.

*Behavioural effects.* After a single administration of chlorpromazine the cats became quiet, withdrawn and ataxic. The nictitating membranes were relaxed; the animals were not hypothermic when killed. A single injection of thioridazine (10 mg/kg) had little apparent effect initially, but 4 hr after injection the animals were quiet, walked clumsily and had relaxed nictitating membranes. The high dose of thioridazine (50 mg/kg) caused relaxation of the nictitating membranes and anal sphincter and diarrhoea; the cats were not hypothermic when killed. Cats treated with thioridazine were less quiet than after chlorpromazine, showing periods of calling and walking about the cage, and one cat treated with the large dose had pronounced rigidity of all limbs and tremor.

Treatment with phenothiazine drugs was prolonged in an attempt to produce conditions similar to those of patients showing Parkinsonism-like side-effects on chronic phenothiazine treatment. Trifluoperazine was given subcutaneously; this caused ulceration at the site of injection in some animals. For this reason, the other drugs were given orally, in capsules. All drugs were given in doses sufficient to cause an observable degree of tranquillization, without causing loss of weight or other noticeable deterioration in condition. No signs of tremor, rigidity, plasticity or anything that could be described as a Parkinsonian syndrome were observed. The animals moved little of their own volition, but were quite capable of normal movement. Any aggression or fear in response to handling or administration of drugs remained unchanged throughout treatment. Thioridazine produced side-effects, the animals suffering from diarrhoea, which prevented a higher dose from being used; some of the treated animals had relaxed nictitating membranes.

TABLE 3

THE EFFECT OF SOME PHENOTHIAZINE TRANQUILLIZERS ON THE MEAN CONTENTS OF SOME AMINES AND THEIR ACID METABOLITES IN BRAIN TISSUE

See Table 2 for detailed caption. All experiments were on cats

| Drug | Dose (mg/kg) and duration of treatment | Mean tissue content ($\mu$g/g fresh tissue) of | | | | |
|---|---|---|---|---|---|---|
| | | Noradrenaline | Dopamine | Homovanillic acid | 5-Hydroxy-tryptamine | 5-Hydroxyindol-3-yl-acetic acid |
| *Acute experiments* | | | | | | |
| Chlorpromazine | 10 s.c., 4 hr | 2·0±0·25 (5) | 4·6±0·4 (9)‡** | 3·6±0·4 (5)‡* | 0·27±0·04 (5) | 0·18±0·01 (5) |
| Control | — | 1·6±0·19 (5) | 6·7±0·5 (8) | 2·0±0·4 (5) | 0·24±0·03 (5) | 0·28±0·07 (5) |
| Thioridazine | 10 s.c., 4 hr | 2·0±0·15 (4)** | 6·2±0·3 (4) | 3·7±0·3 (4)‡** | 0·23±0·07 (4) | 0·28±0·09 (4) |
| Thioridazine | 50 i.p., 4 hr | 1·3±0·13 (4)‡** | 7·0±0·8 (4) | 6·0±1·2 (4)‡* | 0·12±0·003 (3)†* | 0·33±0·08 (3) |
| Control | — | 2·7±0·08 (4) | 7·4±0·9 (4) | 2·1±0·1 (4) | 0·21±0·03 (4) | 0·19±0·03 (4) |
| Total controls | — | 2·1±0·21 (9) | 6·9±0·4 (12) | 2·1±0·2 (9) | 0·22±0·02 (9) | 0·24±0·04 (9) |
| *Chronic experiments* | | | | | | |
| Chlorpromazine | 20 oral, 14 days | 1·4±0·08 (4) | 5·3±0·3 (4) | 3·1±0·2 (4)‡** | 0·32±0·05 (4) | 0·15±0·06 (4) |
| Control | — | 1·8±0·10 (4) | 6·6±0·9 (4) | 2·1±0·2 (4) | 0·37±0·03 (4) | 0·26±0·06 (3) |
| Trifluoperazine | 8 s.c., 12 days | 1·8±0·12 (4) | 5·5±0·9 (4) | 3·6±0·2 (4)‡** | 0·23±0·03 (4) | 0·30±0·05 (4) |
| Control | — | 1·8±0·14 (4) | 6·8±0·1 (4) | 2·2±0·4 (4) | 0·19±0·05 (4) | 0·34±0·07 (4) |
| Thioproperazine | 100 oral, 14 days | 1·6, 1·7 | 5·7±0·4 (3) | 3·5±0·4 (3)‡* | 0·28±0·04 (3) | 0·16±0·03 (3) |
| Control | — | 1·4±0·09 (3) | 3·5±0·9 (3)† | 1·6±0·5 (3) | 0·29±0·03 (3) | 0·25±0·03 (3) |
| Thioridazine | 15 oral, 14 days | 1·6±0·04 (4) | 8·3±0·3 (4) | 2·4±0·3 (4) | 0·25±0·02 (4) | 0·17±0·006 (4) |
| Control | — | 1·4±0·05 (3) | 8·7±1·2 (3) | 2·6±0·2 (3) | 0·34±0·06 (3) | 0·15±0·004 (3) |
| Total controls | — | 1·6±0·07 (14) | 6·5±0·6 (14) | 2·1±0·2 (14) | 0·29±0·03 (14) | 0·24±0·03 (14) |

*Other drugs*

A number of other drugs were examined before and during the course of the above experiments. The first four were used to test the methods; the remaining three in order to investigate drugs used clinically to alleviate Parkinsonism. The results are given in Table 4.

Dopa, the amino acid precursor of dopamine, was used to increase dopamine metabolism. It was given intraperitoneally to cats; in dogs anaesthetized with pentobarbitone it was infused slowly into one carotid artery, the other having been tied off. In both species, the dopamine content of the caudate nucleus increased, and so did the homovanillic acid content in dogs. In cats the homovanillic acid content did not rise, due probably to the short duration of the experiment.

Reserpine (2 mg/kg) reduced the content of all amines in the cat brain and caused a corresponding increase in the two acid metabolites. Guanethidine given to cats had no appreciable effect on catechol amine or homovanillic acid contents. Pentobarbitone anaesthesia had no effect on the brain amines or the acids.

Atropine given in a large dose (25 mg/kg, intraperitoneally) to cats had no effect on amines or their metabolites. It produced pronounced peripheral and mild behavioural effects, the animals remaining quiet when undisturbed but later becoming excitable when handled.

Diethazine (50 mg/kg) and caramiphen (1 mg/kg) had no effect on brain amines and metabolites when given to dogs as a single dose. No behavioural effects were observed.

## DISCUSSION

The aim of the present study was to investigate the effect of drugs on the concentrations in the brain of certain amines and their major metabolites in an attempt to see whether there was any correlation between the behavioural effect of the drug and changes in the content or rate of turnover of an amine in the brain. An increase in the rate of turnover of dopamine was suspected to have taken place whenever an increase in the content of homovanillic acid was observed, particularly when this occurred without a corresponding increase in the metabolite derived from 5-hydroxytryptamine. However, the possibility that a restriction on the outflow of acidic substances from the brain can be produced by a drug cannot be discounted especially when more than one acidic metabolite is increased.

The effects of the drugs which cause central sympathetic excitation on the concentration of the amines in the brain confirm and extend earlier observations and once again illustrate the different responses that are observed with different species. The changes observed with these drugs in the 5-hydroxytryptamine concentration were increases after morphine and $\beta$-tetrahydronaphthylamine and a fall after dexamphetamine. None of these effects were very pronounced. Previous authors (Brodie *et al.*, 1956, and Paasonen & Giarman, 1958, using other species, and Maynert, Klingman & Kaji, 1962, using the dog) found morphine ineffective and dexamphetamine was a depleting agent only in very high doses (Paasonen & Vogt, 1956). Whereas the concentration of dopamine was only exceptionally affected in the cat by drugs which cause central sympathetic excitation, that of homovanillic acid was raised by four of the five drugs tested. The metabolism of dopamine in the caudate nucleus of the cat, like that of the noradrenaline in the hypothalamus of this species, is peculiarly sensitive to drug action. This is illustrated by the fact that the two

TABLE 4

THE EFFECT OF SOME OTHER DRUGS ON THE MEAN CONTENTS OF SOME AMINES AND THEIR ACID METABOLITES IN BRAIN TISSUE

See Table 2 for details. I.c.a., Intracarotid arterial. ø 5-Hydroxytryptamine and 5-hydroxyindol-3-ylacetic acid were measured in the massa intermedia of the thalamus

| Species | Drug | Dose (mg/kg) and duration of treatment | Mean tissue content (μg/g fresh tissue) of | | | | |
|---|---|---|---|---|---|---|---|
| | | | Noradrenaline | Dopamine | Homovanillic acid | 5-Hydroxy-tryptamine | 5-Hydroxyindol-3-ylacetic acid |
| Cat | Dopa | 300 i.p., 30 min | — | 8·1, 11·7* | 1·1, 1·4 | — | — |
| | Reserpine | 2 i.p., 2 hr | 1·4, 1·8 | <1·0 (5)‡** | 5·1±0·5 (5)‡** | 0·33, 0·38ø | 0·47, 0·70ø |
| | Control | — | 3·3 | 4·9±0·7 (4) | 2·0±0·5 (4) | 0·98ø | 0·16ø |
| | Guanethidine | 50 i.p., 4 hr | 2·1, 2·3 | 7·8, 6·3 | 2·1, 4·3 | — | — |
| | Control | — | 1·4 | 7·0 | 4·0 | — | — |
| | Pentobarbitone | 50 i.p., 0·5–1 hr | 1·4, 1·9 | 9·5, 11·7 | 1·5, 3·2 | 0·18, 0·29 | 0·10, 0·23 |
| | Control | — | 1·9, 2·0 | 9·4, 11·1 | 2·7, 3·3 | 0·33, 0·44 | 0·36, 0·42 |
| | Atropine | 25 i.p., 3 hr | 1·5, 2·2 | 7·6, 9·9 | 2·1, 2·3 | 0·23, 0·25 | 0·23, 0·28 |
| | Control | — | 2·0 | 8·9 | 2·7 | 0·25 | 0·25 |
| | Total controls | — | 2·1±0·3 (5) | 6·9±1·0 (8) | 2·6±0·4 (8) | — | — |
| Dog | Dopa | 15 i.c.a., 1–1·5 hr | — | 6·6±0·7 (3)** | 17·1±1·5 (3)†* | — | — |
| | Control | — | — | 2·7±0·3 (4) | 10·0±1·2 (4) | — | — |
| | Diethazine | 50 s.c., 4·5 hr | 1·4, 1·6 | 5·8, 6·3 | 12·8, 13·8 | 0·28, 0·30 | 0·17, 0·22 |
| | Caramiphen | 1 s.c., 4·5 hr | 1·4, 1·5 | 8·0, 10·0 | 13·2, 13·5 | 0·27, 0·28 | 0·14, 0·22 |
| | Control | — | 1·1, 1·8 | 6·0, 7·2 | 11·6, 14·8 | 0·30, 0·35 | 0·18, 0·23 |
| | Total controls | — | — | 4·0±0·9 (6) | 11·4±1·4 (6) | — | — |

drugs ($\beta$-tetrahydronaphthylamine and dexamphetamine) which were tested in cats and dogs increased the homovanillic acid in the cat only. If one wished to correlate the behavioural effect of the two related drugs, dexamphetamine and ephedrine, with their different actions on the metabolism of dopamine in the cat, the only clear sign produced by dexamphetamine and not by ephedrine was the stereotyped forced motor activity.

Oxotremorine is the active metabolite formed in the liver from tremorine (1,4-pyrrolidine-2,3-butyne; Cho, Haslett & Jenden, 1961; Welch & Kocsis, 1961), a substance reported to cause tremor in mice, rats and dogs similar to Parkinsonian tremor (Everett, 1961). In cats, an intense, co-ordinated excitation was produced with little or no tremor, and this was accompanied by an increase in the homovanillic acid in the caudate nucleus.

The increase in homovanillic acid in the caudate nucleus frequently occurs together with a loss of noradrenaline from the hypothalamus. This points to the possibility that under these conditions both dopamine and noradrenaline are being utilized to a greater extent than normal. Since the synthesis of dopamine is faster than that of noradrenaline (Holzer & Hornykiewicz, 1959; Udenfriend & Zaltzman-Nirenberg, 1963), it is not surprising that the dopamine content of the tissue is unchanged whereas that of noradrenaline is reduced. One might expect that under these conditions not only the acid metabolite of dopamine, homovanillic acid, but also the corresponding metabolite of noradrenaline, vanillylmandelic acid, would be raised in those tissues in which the metabolism of noradrenaline is increased. The investigation of this question has not yet been possible because the methods for determining vanillylmandelic acid are not yet sufficiently sensitive.

The phenothiazine derivatives selected for examination were chlorpromazine, trifluoperazine, which causes a high incidence of Parkinsonian side-effects in man (Ayd, 1961), thioridazine, for which a low incidence of these side-effects has been reported (Cole & Clyde, 1961), and thioproperazine, which has a potent catatonic action in rats (Leslie & Maxwell, 1964). Chlorpromazine has been reported to increase the acid metabolites of dopamine in the brain of the rabbit (Andén *et al.*, 1964). Though evidence was obtained in the present work that all four drugs are able to affect the metabolism of dopamine, the effect of thioridazine in the doses used differed from that of the other drugs. After a single dose of thioridazine the caudate nucleus was not depleted of dopamine as was observed with chlorpromazine. After repeated administration any changes in the content of homovanillic acid which were produced initially had disappeared, whereas they persisted with the other three phenothiazine derivatives. In addition, the acute administration of thioridazine caused a depletion of the hypothalamic noradrenaline, an effect which was not seen with chlorpromazine. After thioridazine, the incidence of Parkinsonism is reported to be much lower than after the other phenothiazine drugs used in this work. The observation that it affects amine metabolism in the brain differently from its congeners may have some bearing on this property.

The experiments also show that drugs may have similar behavioural effects but different biochemical actions on amines in the brains of different species; furthermore, that drugs as closely related chemically as the phenothiazine tranquillizers may each have characteristic behavioural and biochemical effects. It is only possible to conclude that there are no simple rules by which the behavioural effect of a drug can be correlated with changes in the metabolism of amines in the brain.

SUMMARY

1. The effects of sixteen drugs on the content of certain amines and their metabolites in brain tissue were studied in an attempt to see whether drugs with central effects caused any change in amine metabolism.

2. The contents of noradrenaline in the hypothalamus and dopamine in the caudate nucleus of cats, dogs and rabbits, of homovanillic acid in the caudate nucleus of cats and dogs, and of 5-hydroxytryptamine and 5-hydroxyindol-3-ylacetic acid in the thalamus of cats and the caudate nucleus of dogs, were measured fluorimetrically following chromatographic separation or solvent extraction.

3. Of those drugs which cause central sympathetic stimulation or " excitement " in cats, morphine (30 to 50 mg/kg), β-tetrahydronaphthylamine (30 mg/kg) and dexamphetamine (10 mg/kg) lowered the noradrenaline content, but only dexamphetamine lowered the dopamine content. All three drugs and also oxotremorine (0.38 mg/kg) increased the homovanillic acid concentration. Morphine and β-tetrahydronaphthylamine slightly increased the 5-hydroxytryptamine content whereas dexamphetamine slightly reduced it; morphine caused a small rise in the 5-hydroxyindol-3-ylacetic acid content. Ephedrine (50 mg/kg) had no effect on any amine or metabolite content.

4. In dogs, β-tetrahydronaphthylamine (30 mg/kg) and dexamphetamine (10 mg/kg) lowered hypothalamic noradrenaline and dexamphetamine lowered the 5-hydroxytryptamine content. No effect on dopamine metabolism was seen. In rabbits, morphine (15 to 80 mg/kg), β-tetrahydronaphthylamine (15 mg/kg) and dexamphetamine (15 mg/kg) produced no change in dopamine level, but dexamphetamine reduced the hypothalamic noradrenaline.

5. Of the phenothiazine derivatives tested in cats, chlorpromazine (10 mg/kg) and thioridazine (10 to 50 mg/kg) given as single injections increased the homovanillic acid but only chlorpromazine lowered the dopamine content. Thioridazine, however, lowered the hypothalamic noradrenaline and, in a large dose, the thalamic 5-hydroxytryptamine content. After treatment for 12 to 14 days with chlorpromazine (20 mg/kg, orally), trifluoperazine (8 mg/kg, subcutaneously) and thioproperazine (100 mg/kg, orally), the homovanillic acid content of the caudate nucleus was increased but the other compounds were not changed; after 14 days' treatment with thioridazine (15 mg/kg, orally) no measurable biochemical change had occurred. Thus thioridazine, which, unlike the other phenothiazine derivatives tested, rarely causes Parkinsonism in man, differs from these compounds in its effects on the metabolism of brain catechol amines.

6. Atropine (25 mg/kg), diethazine (50 mg/kg) and caramiphen (1 mg/kg), drugs associated with the treatment of Parkinsonism, were without effect on brain amine metabolism.

7. No simple correlation between the biochemical and the behavioural effects of the drugs could be found.

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# THE EFFECT OF DRUGS ON THE HOMOVANILLIC ACID CONTENT OF THE CORPUS STRIATUM OF SOME RODENTS

BY

A. V. JUORIO, D. F. SHARMAN AND T. TRAJKOV

*From the Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge*

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The presence of homovanillic acid (4-hydroxy-3-methoxyphenylacetic acid), an acid metabolite of dopamine (3,4-dihydroxyphenylethylamine), in the mammalian brain, particularly in the corpus striatum, has been demonstrated in a number of species (Andén, Roos & Werdinius, 1963a ; Sharman, 1963a ; Bernheimer, 1964 ; Pletscher, Bartholini, Bruderer, Burkard & Gey, 1964). Juorio & Vogt (1965) reported that the striatal region of the rat brain contained very little homovanillic acid and that the level of this acid did not change after the administration of reserpine. In contrast, treatment with reserpine increases the homovanillic acid content of the brain of the rabbit (Andén, Roos & Werdinius, 1964) and of the cat (Sharman, 1963b). It was of interest to examine the response to reserpine in the brains of other small mammals and to reinvestigate the metabolism of the endogenous dopamine in the rat brain.

## METHODS

### *Materials*

These were: L-cysteine hydrochloride (Roche); Dowex 1×2 anion exchange resin 100–200 and 200–400 mesh (Dow Chemical Co.); homovanillic acid (California Corporation for Biochemical Research); hydrochloric acid (Microanalytical Reagent quality); and potassium ferricyanide (Analytical Reagent quality, recrystallized from water). Other reagents were of Analytical Reagent quality. Glass distilled water was used throughout.

The following drugs were used: reserpine (Serpasil, Ciba), either manufacturer's ampoules or a solution prepared from Serpasil powder dissolved in 10% ascorbic acid in water; tetrabenazine, dissolved in a little glacial acetic acid and the solution diluted with 0.9% saline; chlorpromazine hydrochloride, dissolved in 0.9% saline; and DL-3,4-dihydroxyphenylalanine (DL-dopa, L. Light), a suspension prepared in 0.9% saline.

The animals used were guinea-pigs, coypu, albino rats from three colonies and *Meriones* (the gerbil). Rats and *Meriones* were stunned and decapitated; guinea-pigs and coypu were anaesthetized with chloroform and bled. The brain was rapidly removed and the required part dissected out and chilled on ice. When the extraction was not carried out immediately the tissues were stored at −17° C. They did not usually remain at this temperature for longer than 1 hr. The tissue excised was selected for reproducibility of the dissection and varied in the different species as follows: guinea-pig, caudate nucleus and a small amount of the underlying striatal tissue; coypu, caudate nucleus; rat, a block of tissue including the striatum and most of the orbital cortex; and *Meriones,* as for the rat, but with some parietal cortex.

*Extraction of homovanillic acid*

The tissue was homogenized in 2 ml. of 1 N-acetic acid for each g of tissue, using a glass homogenizer. The homogenate was diluted with water to a volume of 5 to 10 ml. 1 N-sodium hydroxide (1 ml./g tissue) was added and the homogenate was mixed thoroughly. After centrifugation the clear supernatant fluid was ready for extraction on a column of anion exchange resin. A small column of Dowex 1 × 2 anion exchange resin was set up as shown in Fig. 1. The resin was washed with 10 ml. of 2 N-hydrochloric acid, followed by 10 ml. of water. It was then converted to the acetate form by means of 10 ml. of 1 N-acetic acid. After washing with a further 15 ml. of water, the column was ready for the passage of the tissue extract. Frequently a small air bubble was trapped just above the resin, preventing the flow of liquid. This was easily removed with a thin glass rod. The tissue extract was filtered into the reservoir above the resin column and the filter paper was washed with water to bring the final volume of liquid in the reservoir to 10 to 20 ml. When all of the extract had passed through the column, the reservoir and column were carefully washed with 10 to 15 ml. of water. Homovanillic acid was eluted with 4 to 4.5 ml. of 0.1 N-hydrochloric acid.

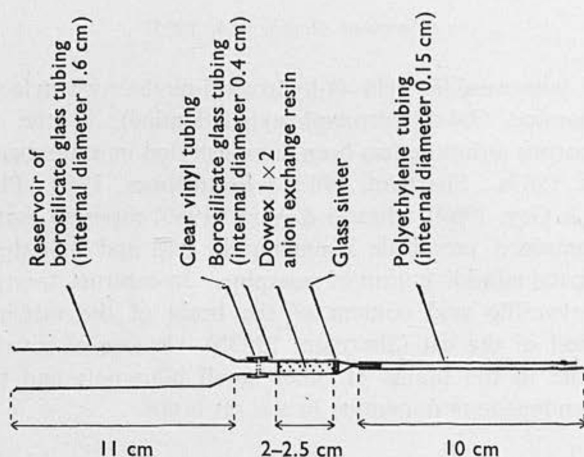


Fig. 1. Diagram of the apparatus used to extract homovanillic acid.

*Fluorimetry*

A slight modification of the method described by Andén *et al.* (1963a) for the estimation of homovanillic acid was used. A portion of the eluate from the resin column was diluted to 1 ml. with water. 1 ml. of a freshly prepared 5 N-ammonium hydroxide solution, containing 20 $\mu$g/ml. of potassium ferricyanide, was then added with mixing. After 4 min the reaction was stopped by the addition of 0.2 ml. of a freshly prepared 0.1% solution of cysteine hydrochloride. A blank determination was made for each eluate by reversing the order of the addition of the last two solutions. The fluorescence developed from authentic homovanillic acid in the presence of the eluate was also determined.

The fluorescence was measured either with an Aminco-Bowman Spectrophotofluorometer or with a Locarte filter fluorimeter. In the former instrument the wavelength of the activating light was 310 m$\mu$ (uncorrected instrumental value). In some experiments a wavelength of 320 m$\mu$ was used for convenience in standardizing the instrument. The fluorescence was measured at a wavelength of 430 m$\mu$ (uncorrected instrumental value) with a Corning 5113 filter in the fluorescence light path. The activating light of the Locarte fluorimeter was passed through two Chance OX7 filters, and the fluorescence light was filtered through a combination of a Corning 5113 (half standard thickness) filter and a Corning 3389 filter. It was found that the results obtained with the filter fluorimeter were somewhat higher than those obtained using the spectrophotofluorimeter unless the method involved the paper chromatographic separation described below. This was due to the

presence of some fluorescing material, not visible in the non-oxidized blank, but also seen in extracts of brain tissue which did not contain homovanillic acid. The results calculated from measurements made with the filter fluorimeter were corrected for this tissue fluorescence. The recovery of authentic homovanillic acid added to tissue homogenates was determined in five series of experiments by three different workers. The mean recoveries ranged from 57 to 71% with an overall mean recovery of 64% (n=67). The standard error of a single observation was ±15.6%.

*Limitations of the method*

The method was developed so that drugs could be quickly screened for their effect on the homovanillic acid content of the brain, and in common with many other simple methods, there are certain limitations to its use. Many acidic substances are adsorbed on to the anion exchange resin under the conditions used. The adsorption of homovanillic acid depends on the nature of other anions present in the extract. For example, the adsorption of homovanillic acid proceeds adequately in the presence of 0.2 M-acetate at *p*H 5.2. A concentration of chloride up to 0.02 M in a solution of 0.14 M-acetate (*p*H 5.2) did not prevent the retention of homovanillic acid, but concentrations of chloride above 0.02 M reduced the recovery, 0.1 M-chloride resulting in a loss of 70%. Homovanillic acid is completely eluted by 8 ml. of 1.0 N-acetic acid or 4 ml. of 0.1 N-hydrochloric acid. These observations indicate that the hydrogen ion contributes to the elution of homovanillic acid from the column. Experiments showed that some derivatives of phenylacetic acid could be separated from the corresponding mandelic acid derivatives, the former being eluted with a more dilute solution of acetic acid. It was found, however, that the development of the fluorescence from homovanillic acid was irregular in the presence of acetic acid. With hydrochloric acid the results are reproducible, but there is a small decrease in the intensity of the fluorescence derived from homovanillic acid. A number of acidic substances, in addition to homovanillic acid, are eluted from the column with 0.1 N-hydrochloric acid. 5-Hydroxyindolylacetic acid was not eluted from the column by 0.1 N-hydrochloric acid, but the eluate would contain any 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxymandelic acid and 4-hydroxy-3-methoxymandelic acid. 3,4-Dihydroxyphenylacetic acid is known to occur in striatal tissue (Andén *et al*., 1963b). When 3,4-dihydroxyphenylacetic acid was present in solutions in a concentration equal to that of homovanillic acid a reduction of 10 to 20% in the fluorescence was observed. Because the eluting acid and possibly other substances present in the eluate are likely to bring about some reduction in the fluorescence, all estimations were calculated against the fluorescence developed from a known amount of homovanillic acid added to a portion of the eluate. In practice, any quenching other than that by the 0.1 N-hydrochloric acid has rarely been seen. The development of a fluorescent compound from homovanillic acid by oxidation with potassium ferricyanide in ammonium hydroxide solution, under the conditions described here, is reasonably specific for this acid. Some other derivatives of 4-hydroxy-3-methoxyphenylethane give rise to a small fluorescence. Of these, only 4-hydroxy-3-methoxymandelic acid is likely to be extracted by this method. This acid has not been detected in brain tissue (Andén *et al*., 1964 ; Sharman, unpublished).

*Identification of homovanillic acid in the tissues*

Tissue extracts, deproteinized with zinc sulphate and sodium hydroxide, or eluates from resin columns were extracted with ethyl acetate. The acids in these extracts were separated by paper chromatography on alkali-washed, hardened filter paper, using a benzene: propionic acid: water mixture as the developing solvent (for details see Sharman, 1963a). Consecutive strips were cut from the whole chromatogram and eluted with water. The fluorescence reaction described above and that described by Sharman (1963a) have been applied to these eluates. The positions of materials on the chromatograms giving rise to a fluorescence were compared with the position of authentic homovanillic acid used as a marker. This was visualized by means of the reaction with diazotized *p*-nitroaniline to give a blue-grey colour. The procedures applied to identify homovanillic acid were also used in some experiments to confirm the estimates made with the quicker but less specific method using the anion exchange resin. For identification, it was frequently necessary to pool the tissues from two to twenty animals.

*Estimation of dopamine*

The dopamine content of rat striatal tissue was estimated fluorimetrically after acetylation of the amine in an eluate from a column of cation exchange resin (Dowex 50 × 8) and condensation with ethylenediamine to form a fluorescent derivative (Laverty & Sharman, 1965a).

## RESULTS

### *Rat*

Groups of rats, obtained from three different sources, were given reserpine, 2 mg/kg. One of these colonies (A) was the same source as the rats used by Juorio & Vogt (1965). The results are given in Table 1. Observations made 4 hr after injection of the drug, the time interval used by Juorio & Vogt (1965), show that, in contrast to animals from colony A, rats from colonies B and C responded with an increase in homovanillic acid in the striatum. The increase seen with rats from colony B was only significant at the 5% level but, in a further series of similar experiments in which the homovanillic acid was estimated after paper chromatography, the effect of reserpine on rats of the same colony was highly significant (Table 2). The results given in Table 2 also confirm that

TABLE 1

THE EFFECT OF DRUGS ON THE CONCENTRATION OF HOMOVANILLIC ACID IN THE CORPUS STRIATUM OF THE RAT

Drugs were given subcutaneously in a single dose : reserpine, 2 mg/kg ; chlorpromazine, 10 mg/kg ; and tetrabenazine, 50 mg/kg. The results are expressed as the means and standard errors of the concentration of homovanillic acid in $\mu$g/g fresh tissue and are corrected for recovery. The number of experiments is given in parentheses. Homovanillic acid was estimated after extraction on an ion exchange column. Significance of difference from control value : $*P<0{\cdot}01$ ; $\dagger P<0{\cdot}05$

| Drug | Duration of experiment (hr) | Homovanillic acid content ($\mu$g/g) for rat colony A | B | C |
|---|---|---|---|---|
| Control | | 0·23±0·05 (14) | 0·23±0·03 (7) | 0·23±0·02 (12) |
| Reserpine | 4 | 0·30±0·08 (7) | 0·43±0·05† (8) | 0·46±0·05* (8) |
| | 6 | 0·60±0·02* (3) | | |
| | 8 | 0·43±0·05* (7) | | 0·44±0·04* (6) |
| | 16 | | | 0·36±0·03* (6) |
| Chlorpromazine | 3 | 0·75±0·12* (4) | | 1·65 ; 0·94 (2) |
| Tetrabenazine | 1·5 | | | 0·77±0·06* (6) |

TABLE 2

THE EFFECT OF DRUGS ON THE CONCENTRATION OF DOPAMINE AND HOMOVANILLIC ACID IN THE CORPUS STRIATUM OF THE RAT

Results are expressed as means and standard errors of the concentration in $\mu$g/g fresh tissue and are corrected for recovery. Homovanillic acid estimations were made after paper chromatographic separation of extracts of pooled tissues. Dopamine estimations were usually made on a portion of the homogenate used for the estimation of homovanillic acid. The number of experiments is given in parentheses. I.p.=intraperitoneal ; s.c.=subcutaneous. Significance of difference from control values : $*P<0{\cdot}01$ ; $\dagger P<0{\cdot}05$

| Colony | Drug | Dose (mg/kg) | Duration of treatment (hr) | Concentration ($\mu$g/g) of Homovanillic acid | Dopamine |
|---|---|---|---|---|---|
| A | Control | | | 0·17±0·02 (5) | 3·0 ±0·3 (8) |
| A | Reserpine | 2 s.c. | 4 | 0·19±0·04 (4) | 0·9 (1) |
| A | Reserpine | 5 i.p. | 4 | 0·10 ; 0·17 (2) | 0·6 ; 0·6 (2) |
| A | Dopa | 200 i.p. | 4 | 3·30 ; 3·30 (2) | 3·2 ±0·4 (6) |
| B | Control | | | 0·20±0·05 (4) | 3·0 ±0·05 (4) |
| B | Reserpine | 2 s.c. | 4 | 0·56±0·03† (4) | |
| B | Tetrabenazine | 50 s.c. | 1·5 | | 0·09±0·01* (4) |

rats from colony A did not show an increase in homovanillic acid 4 hr after the administration of reserpine, even when a larger dose (5 mg/kg) was used. However, rats from colony A did show an increase in homovanillic acid after 6 and after 8 hr. There was no difference between rats from colonies A and C in the response to chlorpromazine (10 mg/kg); a clear increase in homovanillic acid was seen 3 hr after the injection (Table 1). The effect of tetrabenazine was only tested on rats of colony C. The homovanillic acid content of the striatum was increased after 90 min (Table 1).

Homovanillic acid was identified by scanning the whole chromatograms, developed from extracts of the brains of untreated rats (colonies A and C), of rats treated with reserpine (colony A) and of rats given chlorpromazine (colony C). Fig. 2 shows the distribution of material giving rise to fluorescence on chromatograms of extracts from the brains of normal and reserpine-treated rats of colony A. In addition to confirming the presence of homovanillic acid in these tissues, Fig. 2 shows that there is, in normal animals, another fluorescing acidic substance. This fluorescence was seen in alkaline solution, even without the oxidation with ferric chloride, which was used in these experiments to develop the fluorescence from homovanillic acid. The amount of this substance was much reduced after treatment of the animals with reserpine.

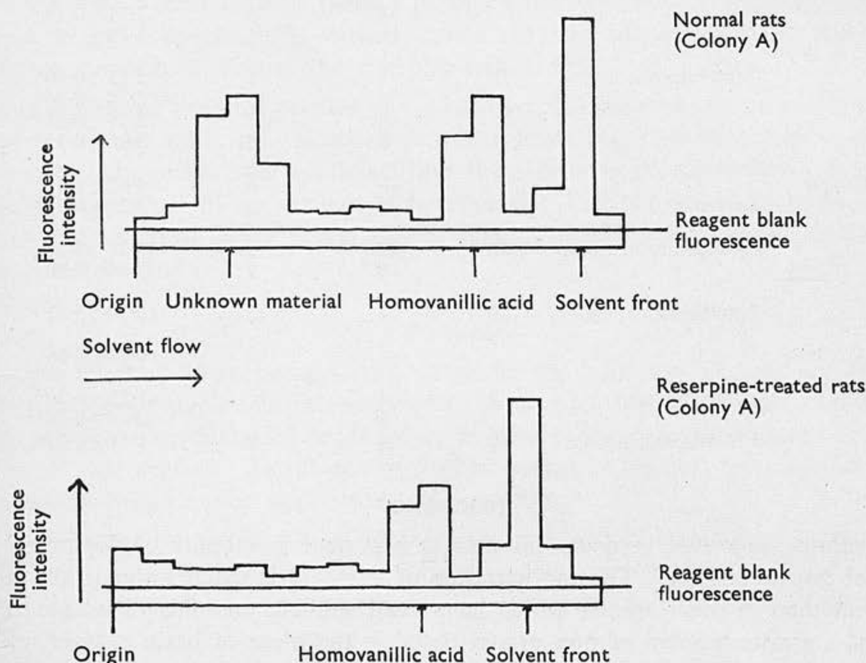


Fig. 2. The distribution of material giving rise to fluorescence on paper chromatograms of acids extracted from the brain of the rat. Fluorescence was developed as described by Sharman (1963a).

*Guinea-pig, coypu and* Meriones

The effects of reserpine, chlorpromazine and tetrabenazine on the content of homovanillic acid in the striatal tissues of the guinea-pig and the coypu are illustrated in Table 3, which also shows the effects of the first two drugs in *Meriones*. In all instances

the drug treatment resulted in an increase in the content of homovanillic acid. The identity of the homovanillic acid in the brains of normal animals was established in all three species.

TABLE 3

THE EFFECT OF DRUGS ON THE CONCENTRATION OF HOMOVANILLIC ACID IN THE CORPUS STRIATUM OF THREE RODENTS

Results (means and standard errors) are expressed as $\mu$g/g fresh tissue and are corrected for recovery. §Homovanillic acid estimations made after paper chromatographic separation. S.c.=subcutaneous; i.p.=intraperitoneal. Significance of difference from control values: $*P<0{\cdot}01$; $\dagger P<0{\cdot}05$

| Species | Drug | Dose (mg/kg) | Duration of treatment (hr) | No. of observations | Homovanillic acid content ($\mu$g/g) |
|---|---|---|---|---|---|
| Guinea-pig | — | | — | 48 | 2·68±0·20 |
| | — | | — | 5 | 2·65±0·60§ |
| | Reserpine, s.c. | 2 | 2 | 11 | 5·36±0·82* |
| | | | 4 | 9 | 6·33±1·02* |
| | | | 8 | 11 | 4·43±0·73* |
| | | | 16 | 5 | 3·41±0·45† |
| | Chlorpromazine, s.c. | 10 | 2 | 4 | 4·78±0·65* |
| | | | 4 | 5 | 4·62±0·35* |
| | | | 8 | 5 | 3·21±0·55 |
| | | | 16 | 5 | 1·78±0·21 |
| | Tetrabenazine, s.c. | 20 | 1 | 11 | 5·23±0·69* |
| | | | 2 | 13 | 5·78±0·56* |
| | | | 4 | 5 | 5·85±0·48* |
| | | | 8 | 5 | 3·96±0·31† |
| Coypu | — | | — | 6 | 4·02±0·35 |
| | Reserpine, i.p. | 4 | 4 | 6 | 7·27±0·87* |
| | Chlorpromazine, i.p. | 10 | 4 | 2 | 11·78 ; 8·58 |
| | | | 8 | 2 | 7·45 ; 7·60 |
| | Tetrabenazine, i.p. | 20 | 4 | 2 | 10·10 ; 5·42 |
| *Meriones* | — | | — | 18 | 0·54±0·09 |
| | Reserpine, s.c. | 2 | 4 | 12 | 1·22±0·26* |
| | | | 8 | 5 | 0·49±0·26 |
| | Chlorpromazine, s.c. | 10 | 4 | 9 | 1·71±0·24* |

## DISCUSSION

The results show that homovanillic acid is a normal metabolite of dopamine in the brains of several rodents. The concentration of this acid is much lower in the striatum of the rat than in other species which have been studied, and this difference is not a result of a greater amount of non-striatal tissue in the piece of brain excised, of which at least one-third was striatal tissue. Significant amounts of homovanillic acid were not detected in cortical tissues. In spite of the large fall in the concentration of dopamine which occurred after reserpine, the increase in homovanillic acid was absent or very small, corresponding at most to about 10% of the fall in the dopamine concentration. This contrasts with the rabbit (Andén *et al.*, 1964) and the cat (Laverty & Sharman, 1965b), in which, 2 hr after the injection of reserpine, the homovanillic acid corresponded to approximately 55% and 33% of the missing dopamine. This suggested that the rat either metabolized dopamine through an alternative pathway or that the homovanillic

acid was more rapidly removed from the brain in this species. That the rat brain is capable of building up high concentrations of homovanillic acid was illustrated by the large increase produced by the administration of DL-dopa (Table 2) and confirms in the rat the observations of Carlsson & Hillarp (1962) on the rabbit. Bertler, Falck & Rosengren (1963) have reported that this treatment results in the formation of dopamine in cells in the walls of the blood-vessels in the brain as well as in nervous tissue, and it is not clear what contribution to the total homovanillic acid content estimated is made by the metabolism of the dopamine in the walls of the cerebral blood-vessels. This objection is, however, not valid for the large rise in homovanillic acid seen after chlorpromazine, which presumably occurs exclusively in nervous tissue. Preliminary experiments on pooled brains of reserpine-treated rats did not reveal any increases in 3-methoxytyramine, 4-hydroxy-3-methoxyphenylethanol or 3,4-dihydroxyphenylacetic acid, known metabolites of dopamine. Their formation would be an alternative reason why, in this species, reserpine causes such a small and delayed increase in the homovanillic acid content of the striatum. When tetrabenazine, which depletes dopamine more rapidly than reserpine, is injected into rats there is a large increase in homovanillic acid. This might be due to the saturation of a mechanism which removes the homovanillic acid from nervous tissue. If such a mechanism were more efficient in the rat than in other species, this would explain the low concentration of homovanillic acid in the striatum of normal and reserpine-treated rats.

In the guinea-pig the time courses of the increases of homovanillic acid after reserpine or chlorpromazine were three times shorter than those observed by Andén *et al.* (1964) in the rabbit. In general, the administration of reserpine or chlorpromazine to mammals results in an increase of the content of homovanillic acid in the striatal tissue, but it is apparent that even within a single species quantitive and temporal differences in this response can occur.

## SUMMARY

1. The effect of some tranquillizing drugs on the homovanillic acid content of the striatum has been studied in rat, guinea-pig, coypu and *Meriones*. The administration of reserpine or chlorpromazine produced an increase in the concentration of homovanillic acid in all four species. Tetrabenazine was examined in the first three species ; it also increased the homovanillic acid content of the brain.

2. In the rat the normal level of this acid is low and the increase afer reserpine is small and may be delayed. It is suggested that, in this species, there is a more efficient mechanism for removing homovanillic acid from the brain than in other species.

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# CHANGES IN THE METABOLISM OF 3,4-DIHYDROXYPHENYLETHYLAMINE (DOPAMINE) IN THE STRIATUM OF THE MOUSE INDUCED BY DRUGS

BY

**D. F. SHARMAN**

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# CHANGES IN THE METABOLISM OF 3,4-DIHYDROXYPHENYLETHYLAMINE (DOPAMINE) IN THE STRIATUM OF THE MOUSE INDUCED BY DRUGS

BY

D. F. SHARMAN

*From the Pharmacology Unit, Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge*

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Several drugs have been shown to increase the concentration of 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid ; HVA), a major metabolite of 3,4-dihydroxyphenylethylamine (dopamine), in the striatal tissues of several mammalian species (Andén, Roos & Werdinius, 1963, 1964 ; Sharman, 1963 ; Laverty & Sharman, 1965a ; Juorio, Sharman & Trajkov, 1966). This report is concerned with the application of a screening test for drugs which increase the concentration of homovanillic acid in the striatum using the albino mouse as the test animal. Possible mechanisms by which such an increase can be produced are suggested.

## METHODS

### *Administration of drugs*

The drugs were administered intraperitoneally or, in a few cases, intravenously, dissolved in 0.9% sodium chloride solution with the following exceptions.

Reserpine was injected as the manufacturers' solution.

Tetrabenazine, spiroperidol, haloperidol and triperidol were dissolved in the minimum amount of glacial acetic acid. Dilutions were then made with 0.9% sodium chloride solution.

Probenecid was dissolved in the minimum volume of 1 N-sodium hydroxide and 0.9% sodium chloride solution was then added. If necessary, the *p*H was adjusted to 7–8 with 0.1 N-hydrochloric acid.

$\alpha$-Methyl-p-tyrosine was dissolved in the minimum volume of 1 N-sodium hydroxide and the solution diluted with 0.5 M-disodium hydrogen phosphate solution ; 0.4 N-hydrochloric acid was then added carefully until a slight precipitate of the amino acid was formed. The final concentration of $\alpha$-methyl-p-tyrosine was usually 8 mg/ml.

### *Dissection of tissues*

The mice were stunned and decapitated and the brains rapidly dissected out and placed on a glass plate on ice. The part of the brain used for the estimations of homovanillic acid and of dopamine consisted of the striatum together with some orbital cortex. This piece of tissue was selected for ease of reproducibility in the dissection and was obtained by dividing the brain along the midline and exposing the caudate nucleus through the lateral ventricle. The cortical tissue anterior and dorsal to the caudate nucleus was removed and the striatum together with some orbital cortex obtained with a single cut posterior to the caudate nucleus. For the estimation of 5-hydroxyindol-3-ylacetic acid the whole brain anterior to the pons was used. Pooled tissues from two to three mice were used for the estimation of homovanillic acid and from two mice for the estimation of

5-hydroxyindol-3-ylacetic acid. The dopamine estimations were carried out with the tissue from a single animal. Albino mice of both sexes were used.

*The estimation of homovanillic acid*

The method used to extract and estimate homovanillic acid has been described in detail by Juorio *et al.* (1966). Briefly, the homovanillic acid was adsorbed from the deproteinized tissue extract on to a small column of Dowex 1X2 anion exchange resin. The homovanillic acid was then eluted with 0.1 N-hydrochloric acid and estimated fluorimetrically by a modification of the method of Andén *et al.* (1963) using an Aminco-Bowman Spectrophotofluorometer.

Dopamine was extracted with a column of Dowex 50X8 cation exchange resin as described by Bertler, Carlsson & Rosengren (1958). The dopamine in the eluate from the column was acetylated and estimated fluorimetrically in a Locarte filter fluorimeter after condensation of the acetyl derivative with ethylene diamine (Laverty & Sharman, 1965b). 5-Hydroxyindol-3-ylacetic acid was estimated fluorimetrically as described by Ashcroft & Sharman (1962). All estimations are uncorrected for recoveries.

## RESULTS

### *Effect of drugs on the concentration of homovanillic acid in the striatum*

The effect of a number of drugs on the concentration of homovanillic acid in the striatum of the mouse is shown in Table 1. No difference was found between untreated control animals and those treated with the vehicles in which the drugs were dissolved. All of these values have been combined to give the control value in Table 1.

TABLE 1

THE EFFECT OF DRUGS ON THE CONCENTRATION OF HOMOVANILLIC ACID IN THE STRIATUM OF THE MOUSE

Except where otherwise stated drugs were injected intraperitoneally

* Significantly different ($P<0{\cdot}01$) from control value. A single observation showing an increase of 0·24 μg/g above the control value can be taken to indicate a significant increase

| Drug | Dose (mg/kg) | Duration of treatment (hr) | Concn. of homovanillic acid (μg/g tissue±s.e.m.) No. of observations in parentheses |
|---|---|---|---|
| Control | | | 0·31±0·01 (76) |
| *Tranquillizing drugs* | | | |
| Body temperature maintained where necessary | | | |
| Chlorpromazine HCl | 2·5 | 3 | 0·45 ; 0·31 |
| | 5·0 | 3 | 0·70±0·16 (10)* |
| | 10·0 | 3 | 0·91±0·15 (3)* |
| Thioridazine HCl | 10·0 | 3 | 0·49 ; 0·49 |
| Prothipendyl HCl | 10·0 | 3 | 0·30 ; 0·28 |
| | 50 | 3 | 0·50 |
| | 100 | 3 | 0·69 ; 0·81 |
| Chlorprothixene | 10 | 3 | 1·28±0·08 (3)* |
| Reserpine | 2·5 | 3 | 0·54 ; 0·36 |
| | 5 | 3 | 0·48±0·01 (3) |
| Tetrabenazine | 50 | 1·5 | 0·73±0·02 (3)* |
| Spiroperidol | 0·1 | 2 | 0·72 ; 0·86 |
| | | 3 | 0·66±0·04 (9)* |
| | 0·25 | 3 | 0·48 ; 0·72 |
| | 0·5 | 3 | 0·91±0·07 (4)* |
| Haloperidol | 0·5 | 2 | 0·50 ; 0·92 |
| Triperidol | 0·5 | 2 | 1·01 ; 0·82 |
| Chlordiazepoxide | 50 | 1 | 0·43 ; 0·41 |
| | 100 | 1 | 0·49±0·06 (4)* |
| | 200 | 1 | 0·54 ; 0·66 |

TABLE 1—*continued*

| Drug | Dose (mg/kg) | Duration of treatment (hr) | Concn. of homovanillic acid ($\mu$g/g tissue $\pm$ s.e.m.) No. of observations in parentheses |
|---|---|---|---|
| *Excitant drugs* | | | |
| Methylphenidate | 20 | 3 | 0·18 ; 0·10 |
| | 50 | 2 | 0·31 ± 0·03 (3) |
| $\beta$-Tetrahydronaphthylamine | 50 | 2 | 0·91 ± 0·18 (3)* |
| *Morphine and associated compounds* | | | |
| Morphine HCl | 45 mg/kg in two doses at 2-hr interval Total duration 4 hr | | 0·42 ± 0·07 (3) |
| M.99 | 0·1 | 1 | 0·53 ; 0·58 |
| | | 2 | 0·88 ± 0·06 (3)* |
| | | 4 | 0·79 ± 0·12 (3)* |
| | | 8 | 0·27 ± 0·01 (3) |
| Body temperature maintained | 0·1 | 2 | 0·58 ± 0·07 (3)* |
| | 0·2 | 2 | 0·84 ; 0·76 |
| M.285 | 0·5 | 2 | 0·32 ± 0·02 (4) |
| Phencyclidine HCl | 5 | 1 | 0·36 ; 0·32 |
| | | 4 | 0·25 ; 0·30 |
| | 10 | 1 | 0·34 ; 0·42 |
| *Central cholinomimetic drugs* | | | |
| These mice were pretreated with atropine methyl bromide 2 mg/kg s.c. | | | |
| Arecoline HBr | 2 I.V. | 2 | 0·31 ; 0·30 |
| | 6 I.V. | 2 | 0·28 |
| | 10 I.V. | 2 | 0·30 |
| Pilocarpine HCl | 2 I.V. | 2 | 0·27 |
| | 4 I.V. | 2 | 0·34 ; 0·34 |
| *Anti-depressant drugs* | | | |
| Pargyline HCl | 400 | 0·5 | 0·20 ; 0·18 |
| | | 1 | 0·20 ; 0·26 |
| | | 4 | 0·08 ; 0·06 |
| Imipramine | 20 | 4 | 0·40 ± 0·09 (4) |
| Desmethylimipramine | 20 | 4 | 0·23 ; 0·25 |
| *Other drugs* | | | |
| Probenecid | 200 | 3·5 | 0·75 ; 0·78 |
| | 270 | 3·5 | 1·31 ± 0·10 (4)* |
| Zoxazolamine | 90 mg/kg divided into 3 doses at 1-hr intervals Total duration 2·5 hr | | 0·25 ± 0·02 (3) |
| Tolazoline | 35 mg/kg divided into 4 doses at 30-min intervals Total duration 2 hr | | 0·28 ± 0·02 (5) |
| Bulbocapnine HCl | 40 | 0·5 | 0·51 ± 0·05 (4)* |
| Benzhexol HCl | 50 I.V. | 2 | 0·43 ; 0·40 |

The large increases caused by most of the tranquillizing drugs are in agreement with the observations of Andén *et al.* (1964) and Roose (1965) on the rabbit, Laverty & Sharman (1965a) on the cat and Juorio *et al.* (1966) on four rodents. The small increase seen after chlordiazepoxide was seen only after doses of this drug which produce a loss of the righting reflexes.

Of the two excitant drugs, only $\beta$-tetrahydronaphthylamine produced an increase in the level of homovanillic acid, an effect also seen in the cat (Laverty & Sharman, 1965a). These authors also observed that morphine elevated the homovanillic acid in the cat. When tested in the mouse morphine did not show this effect even after 45 mg/kg. However, the very active morphine-like compound M.99 (6,14-endoetheno-7-(2-hydroxy-

2-pentyl)-tetrahydro-oripavine hydrochloride) produced a clear increase after a dose of only 0.1 mg/kg. M.285 (N-cyclopropylmethyl-6,4-endoetheno-7-(2-hydroxy-2-propyl)-tetrahydro-nororipavine hydrochloride), which has strong nalorphine-like properties, did not produce an increase in homovanillic acid when 0.5 mg/kg were given. With a larger dose (1 mg/kg) a possible small increase in the homovanillic acid was seen (Table 2).

TABLE 2

THE EFFECT OF PRETREATMENT ON DRUG-INDUCED INCREASES IN THE HOMOVANILLIC ACID CONCENTRATION IN THE STRIATUM OF THE MOUSE

Except where stated otherwise all drugs were injected intraperitoneally

* Significant difference ($P<0 \cdot 01$) from M.99 alone
† Significant difference ($P<0 \cdot 01$) from all controls (Table 1); not significantly different from parallel control values

| Drug | Dose (mg/kg) | Duration of treatment (hr) | Pretreatment 15–60 min before (dose in mg/kg) | | Concn. of homovanillic acid (μg/g tissue ± s.e.m.) No. of observations in parentheses |
|---|---|---|---|---|---|
| Chlorpromazine HCl | 5 | 3 | | | 0·70±0·05 (10) |
| | | | Diethazine | 10 | 0·66 ; 0·77 |
| | | | Caramiphen | 10 | 0·76 ; 0·61 |
| | | | Desmethylimipramine | 20 | 0·78±0·06 (3) |
| | | | Imipramine | 20 | 0·93±0·01 (4) |
| | | | Methylphenidate | 20 | 0·80 ; 0·72 |
| | | | Methylphenidate | 50 | 0·60 ; 0·65 |
| Spiroperidol | 0·1 | 2 | | | 0·70±0·03 (11) |
| | | | M.285 | 1 | 0·63±0·04 (8) |
| Spiroperidol | 0·1 | 3 | | | 0·66±0·04 (9) |
| | | | Phencyclidine | 5 | 0·59 ; 0·67 |
| | | | Benzhexol | 10 I.V. | 0·51 ; 0·81 |
| M.99 | 0·2 | 2 | | | 0·75±0·06 (7) |
| | | | M.285 | 1 | 0·53±0·04 (8)* |
| M.285 | 1 | 2 | | | 0·45±0·04 (7)† |
| Control animals | (Estimations made in parallel with above three sets of results) | | | | 0·37±0·05 (6) |

Inhibition of monoamine oxidase by pargyline resulted in a fall in the homovanillic acid level, an effect seen in the rabbit after treatment with the monoamine oxidase inhibiting drug nialamide (Andén *et al.*, 1963). The two other anti-depressant drugs tested, which are not inhibitory to monoamine oxidase, had no significant effect on the concentration of homovanillic acid. The drugs causing central cholinergic stimulation were also ineffective in this respect. Of the other drugs tested, probenecid is of interest as an inhibitor of organic acid transport in the renal tubules ; it produced a large elevation of the level of homovanillic acid in the striatum. An increase in the concentration of 5-hydroxyindol-3-ylacetic acid in the brain of the rat after treatment with probenecid has been reported by Neff, Tozer & Brodie (1964). Zoxazolamine has uricosuric properties which can be additive with those of probenecid, and is a centrally acting muscle relaxant, but it did not affect the concentration of homovanillic acid in striatal tissues.

*Antagonism of drug induced increases in the concentration of homovanillic acid*

Because the major tranquillizing drugs are known to produce side effects which resemble Parkinson's disease, some drugs which are used to treat this disease were tested to see if they would prevent the increase in homovanillic acid produced by chlorpromazine and spiroperidol. The drugs were administered intraperitoneally 15–60 min before the tranquillizing drug.

The results are shown in Table 2 and they indicate that diethazine and caramiphen do not prevent the increase in homovanillic acid produced by chlorpromazine and that benzhexol is ineffective against spiroperidol. Imipramine, desmethylimipramine and methylphenidate were also inactive. Table 2 also shows that M.285, in a dose that blocks the behavioural effects of M.99 administered 15 min later, can partially antagonize the increase in homovanillic acid produced by the latter drug. The same dose of M.285 did not change the homovanillic acid increase after spiroperidol (0.1 mg/kg).

*Investigation of the mechanisms by which drugs can elevate the concentration of homovanillic acid in the striatum*

Five of the drugs found to increase the homovanillic acid concentration in the striatum of the mouse were selected for further investigation into the mechanisms by which they produce this effect. These were spiroperidol, M.99, β-tetrahydronaphthylamine, probenecid and bulbocapnine.

(a) *Behavioural effects*

These five drugs produce very different behavioural effects in the mouse.

Spiroperidol in a dose of 0.1 mg/kg had little effect on the gross behaviour of the mice. They remained fairly active but frequently showed ptosis. With a larger dose (0.5 mg/kg) the animals became inactive but did not collapse.

The response to M.99 (0.2 mg/kg) was extremely variable. A pronounced Straub effect on the tail was the first sign of the action of the drug. The animals then became excited and moved erratically about the cage. A rigid catalepsy then developed and in many but not all of the experiments the animals lost their righting reflexes. There was a pronounced fall in temperature.

β-Tetrahydronaphthylamine caused piloerection on the back of the neck and excitement.

Probenecid (200 mg/kg), had no obvious effect on the behaviour of the mice.

Bulbocapnine (40 mg/kg) caused a flaccid catalepsy which wore off after one hour. None of these behavioural effects was prevented by the administration of α-methyl-p-tyrosine.

(b) *Effects on the rate of utilization of dopamine*

The effects of these five drugs on the concentrations of dopamine and homovanillic acid in the striatum of the mouse were studied after treatment with the drugs alone and after pretreatment with α-methyl-p-tyrosine (80 mg/kg). This amino acid competitively inhibits the enzyme tyrosine hydroxylase (Nagatsu, Levitt & Udenfriend, 1964; Udenfriend, Zaltzman-Nirenberg & Nagatsu, 1965), which catalyses the rate-limiting step

in the synthesis of dopamine *in vivo*. Spector, Sjoerdsma & Udenfriend (1965) have shown that the administration of this amino acid results in a fall of the dopamine concentration in the caudate nucleus and suggested that the rate of the decrease in dopamine concentration in a tissue, after treatment with α-methyl-p-tyrosine, would reflect the turnover rate of the dopamine in that tissue.

The results obtained in this series of experiments are given in Tables 3 and 4. A maximum inhibition of the tyrosine hydroxylase was effected in these experiments because a larger dose of α-methyl-p-tyrosine (160 mg/kg) did not produce a larger fall in the concentration of dopamine than that following a dose of 80 mg/kg. Table 3 shows that of the five drugs only β-tetrahydronaphthylamine changes the dopamine concentration when given alone. In a dose of 30 mg/kg this drug caused a significant increase in the concentration of dopamine in the striatum of the mouse. Table 3 also shows that after α-methyl-p-tyrosine, the administration of spiroperidol in doses of 0.1 mg/kg and 0.5

TABLE 3

THE EFFECT OF DRUGS ON THE DECREASE IN THE CONCENTRATION OF THE DOPAMINE IN THE STRIATUM OF THE MOUSE PRODUCED BY THE ADMINISTRATION OF α-METHYL-p-TYROSINE

α-Methyl-p-tyrosine (80 mg/kg) administered 15 min before drug

* Significant difference ($P<0{\cdot}01$) from corresponding control value
† Significant difference ($P<0{\cdot}01$) from control animals not treated with α-methyl-p-tyrosine

| Drug | Dose (mg/kg) | Duration of treatment (hr) | Dopamine concn. (μg/g tissue ± s.e.m.) No. of observations in parentheses: Drug alone | After α-methyl-p-tyrosine |
|---|---|---|---|---|
| Control | | | 3·61±0·14 (20) | 1·86±0·05 (25)† |
| Spiroperidol | 0·1 | 2 | 2·91±0·15 (6) | 1·49±0·11 (12)* |
| | 0·5 | 2 | 2·67±0·09 (6) | 1·16±0·06 (6)* |
| β-Tetrahydro-naphthylamine | 30 | 2 | 4·35±0·13 (17)* | 2·01±0·11 (6) |
| M.99 | 0·2 | 2 | 3·17±0·08 (8) | 1·98±0·10 (6) |
| Bulbocapnine HCl | 40 | 1·25–1·5 | 2·90±0·22 (6) | 1·83±0·13 (9) |
| Probenecid | 200 | 2 | 2·85±0·20 (6) | 2·07±0·21 (9) |

TABLE 4

THE EFFECT OF α-METHYL-p-TYROSINE ON THE DRUG-INDUCED INCREASE IN THE LEVEL OF HOMOVANILLIC ACID IN THE STRIATUM OF THE MOUSE

α-Methyl-p-tyrosine (80 mg/kg) administered 15 min before drug

* Significant difference ($P<0{\cdot}01$) from corresponding control value
† Significant difference ($P<0{\cdot}01$) from control animals not treated with α-methyl-p-tyrosine

| Drug | Dose (mg/kg) | Duration of treatment (hr) | Concn. of homovanillic acid (μg/g tissue ± s.e.m.) No. of observations in parentheses: Drug alone | After α-methyl-p-tyrosine |
|---|---|---|---|---|
| Control | | | 0·31±0·01 (76) | 0·18±0·01 (10)† |
| Spiroperidol | 0·1 | 2 | 0·70±0·03 (11)* | 0·22±0·01 (6) |
| | 0·5 | 2 | 1·04±0·05 (5)* | 0·34±0·03 (7)* |
| β-Tetrahydro-naphthylamine | 30 | 2 | 0·55±0·05 (12)* | 0·19±0·01 (4) |
| M.99 | 0·2 | 2 | 0·75±0·06 (7)* | 0·26±0·02 (10) |
| Bulbocapnine HCl | 40 | 1·25–1·5 | 0·70±0·06 (12)* | 0·21±0·01 (4) |
| Probenecid | 200 | 2 | 0·90±0·02 (6)* | 0·47±0·03 (8)* |

mg/kg results in a significantly larger fall in the concentration of dopamine than occurs after the amino acid alone. The other four drugs did not change the fall in dopamine concentration caused by the α-methyl-p-tyrosine.

*The specificity of the dopamine estimations.* To confirm that the apparent increase in the dopamine concentration seen after β-tetrahydronaphthylamine was due to dopamine and not to a metabolite of the drug, striatal tissues from treated mice were extracted as for the estimation of dopamine. After the acetylation step in the procedure the solution was extracted with dichloromethane and the material in this extract subjected to paper chromatographic separation as described by Laverty & Sharman (1965b). Fluorescence was only found on the chromatogram in the region of triacetyldopamine, and this was present in an intensity which corresponded with the increased amount found by direct estimation in the tissue. Furthermore, no increase in dopamine was detected in the hypothalamic region of the mouse brain after treatment with β-tetrahydronaphythylamine, where metabolites of β-tetrahydronaphthylamine, if such were formed, should also be detected.

Udenfriend *et al.* (1965) have shown that a small amount of α-methyl-3,4-dihydroxyphenylalanine can be formed from α-methyl-p-tyrosine *in vitro* by the enzyme tyrosine hydroxylase. If this transformation were to occur *in vivo* then it is possible that the dopamine estimations given in Table 3 would include any α-methyl dopamine formed by decarboxylation of the α-methyl-3,4-dihydroxyphenylalanine. However, no α-methyl dopamine could be detected in extracts of striatal tissue from mice treated with α-methyl-p-tyrosine when these were examined by the methods described above for the identification of dopamine since these allow the separation of dopamine and its methylated derivative α-methyl dopamine. Using these methods it was possible to demonstrate that α-methyl dopamine was formed in striatal tissue 2 hr after the administration of α-methyl-3,4-dihydroxyphenylalanine (80 mg/kg).

(c) *Effects on the concentration of homovanillic acid*

Table 4, in agreement with Table 1, shows that all five drugs can produce a significant increase in the homovanillic acid concentration in the striatum of the mouse. Inhibition of tyrosine hydroxylase by α-methyl-p-tyrosine causes a reduction in the level of the homovanillic acid in this tissue. Furthermore, α-methyl-p-tyrosine given 15 min before the drug reduces the increase in homovanillic acid produced by probenecid and the higher dose of spiroperidol and abolishes that caused by the lower dose of spiroperidol, β-tetrahydronaphthylamine, M.99 and bulbocapnine.

(d) *Effects on 5-hydroxyindol-3-ylacetic acid*

The five drugs were also examined for their effect on the concentration of 5-hydroxyindol-3-ylacetic acid in the brain. The results are given in Table 5. Probenecid was found to cause an increase in the concentration of 5-hydroxyindol-3-ylacetic acid in the brain whereas β-tetrahydronaphthylamine produced a reduction in the concentration of this acid. No effect on the 5-hydroxyindol-3-ylacetic acid level was observed after spiroperidol, M.99 and bulbocapnine.

TABLE 5

THE EFFECT OF DRUGS ON THE CONCENTRATION OF 5-HYDROXYINDOL-3-YLACETIC ACID IN THE BRAIN OF THE MOUSE

* Significantly different ($P<0{\cdot}01$) from control values

| Drug | Dose (mg/kg) | Duration of treatment (hr) | Concn. of 5-hydroxyindol-3-ylacetic acid (μg/g tissue ± s.e.m.) No. of observations in parentheses |
|---|---|---|---|
| *First series of experiments* | | | |
| Control | | | 0·21±0·01 (4) |
| Spiroperidol | 0·5 | 3·5 | 0·24±0·03 (4) |
| Probenecid | 200 | 3·5 | 0·49±0·06 (4)* |
| *Second series of experiments* | | | |
| Control | | | 0·18±0·01 (24) |
| Spiroperidol | 0·5 | 2 | 0·22±0·02 (6) |
| β-Tetrahydronaphthylamine | 30 | 2 | 0·09±0·02 (6)* |
| M.99 | 0·2 | 2 | 0·22±0·03 (6) |
| Bulbocapnine HCl | 40 | 1·25–1·5 | 0·18±0·02 (6) |
| Probenecid | 200 | 2 | 0·35±0·02 (6)* |

## DISCUSSION

The method for estimating homovanillic acid described by Juorio *et al.* (1966) has proved to be sensitive enough to be used in a screening test for drugs which affect the homovanillic acid in the striatum, using the albino mouse as the test animal. This enables several observations to be made when only a limited amount of a drug is available.

The dopamine in the striatum is thought to have a physiological role of its own and the major final metabolite of dopamine in this region of the brain appears to be homovanillic acid. An increased formation of homovanillic acid could follow an increased synthesis of the amine. If the dopamine storage mechanism is not saturated under normal conditions, this increased synthesis would be accompanied by an increase in the amount of dopamine present in the tissue. An inhibitor of dopamine synthesis would abolish drug-induced increases in the concentrations of both dopamine and homovanillic acid. Secondly, an increased requirement for dopamine could result in a compensatory increase in the synthesis of dopamine through a feedback mechanism which maintains the level of the stored amine. Inhibition of the synthesis of dopamine would reduce the increase in homovanillic acid, but the increased requirement for the amine would be reflected in a depletion of the stored dopamine greater than that produced by inhibition of the synthesis of dopamine under normal conditions. When the formation of dopamine is inhibited a small increase in homovanillic acid might be produced by a drug acting in this way but this will depend on the magnitude of the increase in the rate of depletion of the stored amine.

Finally, a drug might also reduce the rate of outflow of homovanillic acid from the brain. After inhibition of the synthesis of dopamine an increase in homovanillic acid would still be seen. If such a drug is not acting specifically on the mechanism which removes homovanillic acid from the brain then a concomitant increase in other acidic substances such as 5-hydroxyindol-3-ylacetic acid should be seen.

These mechanisms are illustrated diagrammatically in Fig. 1.

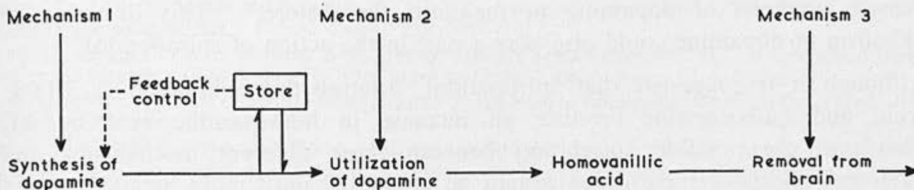


Fig. 1. The sites in the major metabolic pathway of dopamine in the striatum at which a drug might act to produce an increase in the concentration of homovanillic acid.

In the mouse, β-tetrahydronaphthylamine seems to be acting by the first mechanism proposed, increasing the synthesis of dopamine, since both the concentration of the amine and its acid metabolite are increased. Both of these effects are abolished when the synthesis of dopamine is inhibited by α-methyl-p-tyrosine. Because β-tetrahydronaphthylamine does not accelerate the fall in the dopamine concentration caused by α-methyl-p-tyrosine, there is no increase in the requirement for dopamine. The enzyme tyrosine hydroxylase catalyses the rate-limiting step in the synthesis of dopamine and it is tempting to ascribe the action of β-tetrahydronaphthylamine to an effect on this enzyme.

Spiroperidol might be acting on the second mechanism. It appears to act by increasing the turnover of dopamine, since, after inhibition of the synthesis of dopamine there is a faster rate of depletion of the amine in mice treated with this drug than in untreated animals. Only the larger dose of spiroperidol increased the concentration of homovanillic acid after treatment with α-methyl-p-tyrosine. It would further appear that part of the action of this drug is dependent upon an intact synthetic pathway for dopamine.

Probenecid is a typical example of a drug which acts by generally preventing the outflow of acidic substances from the brain. This drug has no effect on the concentration of dopamine when given alone or after α-methyl-p-tyrosine. After the latter treatment, probenecid still produces a large increase in homovanillic acid. This increase is not as large as in the normal animal and reflects a lowering of the turnover rate of dopamine as the " stored " amine is reduced. The general action of probenecid is illustrated by the fact that it was the only drug of the five examined which elevated the concentration of 5-hydroxyindol-3-ylacetic acid in the brain of the mouse. Neff *et al.* (1964) have demonstrated an active system for transporting 5-hydroxyindol-3-ylacetic acid out of the brain of the rat, which can be inhibited by probenecid. The observations presented in this paper confirm the presence of a similar system in the mouse.

On the evidence presented here, the actions of M.99 and bulbocapnine in elevating homovanillic acid do not appear to fit any of the proposed schemes. These drugs did not increase the concentration of dopamine like β-tetrahydronaphthylamine and in the presence of an inhibitor of dopamine synthesis there was no increase in the rate of disappearance of dopamine, as had been seen with spiroperidol. Their action requires an intact synthetic pathway for dopamine, and they do not act by preventing the outflow of homovanillic acid from the brain. A possible explanation of the action of M.99 and bulbocapnine is that they produce a diversion of the dopamine after the amine is formed by the decarboxylation of 3,4-dihydroxyphenylalanine and that it is metabolized before it comes under the influence of the "storage " mechanisms. Such a diversion would tend to reduce the amount of dopamine in the " store " and this, in turn, might lead to an

increased synthesis of dopamine to maintain the "store." This diversion of the metabolism of dopamine could also play a part in the action of spiroperidol.

Although it is suggested that spiroperidol, $\beta$-tetrahydronaphthylamine, M.99, probenecid and bulbocapnine produce an increase in homovanillic acid by different mechanisms, the possible correlation between these different mechanisms and the behavioural effects of these drugs cannot be attempted until more detailed behavioural studies have been made.


SUMMARY

1. A rapid method is described for the detection of drugs which affect the concentration of 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid) in the striatum, using the albino mouse as the test animal.

2. Among the drugs which were found to increase the concentration of homovanillic acid in the striatum of the mouse, spiroperidol, $\beta$-tetrahydronaphthylamine, M.99, bulbocapnine and probenecid were selected for a fuller analysis. This included the effect of these drugs on the fall in brain concentration of 3,4-dihydroxyphenylethylamine (dopamine) elicited by $\alpha$-methyl-p-tyrosine.

3. These observations, and the study of the effects of these drugs on the concentration of 5-hydroxyindol-3-ylacetic acid in the brain, have suggested a number of mechanisms by which drugs can produce an increased concentration of homovanillic acid in the striatum.


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# A DISCUSSION OF THE MODES OF ACTION OF DRUGS WHICH INCREASE THE CONCENTRATION OF 4-HYDROXY-3-METHOXYPHENYLACETIC ACID (HOMOVANILLIC ACID) IN THE STRIATUM OF THE MOUSE

BY

D. F. SHARMAN

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# A DISCUSSION OF THE MODES OF ACTION OF DRUGS WHICH INCREASE THE CONCENTRATION OF 4-HYDROXY-3-METHOXYPHENYLACETIC ACID (HOMOVANILLIC ACID) IN THE STRIATUM OF THE MOUSE

BY

D. F. SHARMAN

*From the Pharmacology Unit, Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge*

(*Received April* 10, 1967)

The administration to rats of probenecid, an inhibitor of the transport of organic acids in the renal tubules, has been shown to cause an increased concentration in the brain of 5-hydroxyindol-3-ylacetic acid, a metabolite of 5-hydroxytryptamine (Neff, Tozer & Brodie, 1964). Sharman (1966) reported a similar response to probenecid in the mouse and observed a concomitant elevation in the striatum of the concentration of 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid), a metabolite of 3,4-dihydroxyphenylethylamine (dopamine). Werdinius (1966) showed that an increase in the concentration of homovanillic acid also occurred in the brain of the rat after treatment with probenecid. From these observations it has been concluded that there is an active transport mechanism for the removal of these acid metabolites from the brain in these two species. This report is concerned with a method which distinguishes between two types of drug action by which an increase of the striatal concentration of homovanillic acid in the mouse can occur, using the response to a standard dose of probenecid.

## METHODS

Female albino mice of a single strain were used. Drugs were administered intraperitoneally, dissolved in a 0.9% w/v solution of sodium chloride. Probenecid was dissolved in the minimum volume of 1N-sodium hydroxide and 0.9% w/v sodium chloride solution was then added. If necessary, the pH of the solution was adjusted to 7–8 with 0.1N-hydrochloric acid. Spiroperidol was dissolved in the minimum volume of glacial acetic acid (10 mg spiroperidol will dissolve in 0.05 ml. glacial acetic acid), the solution was diluted with a little water and final dilutions were made with 0.9% w/v sodium chloride solution. The dissection of the striatal tissues, the extraction and fluorimetric estimation of homovanillic acid were carried out as described by Sharman (1966). The striatal tissue from 2 mice was used for each estimation.

## RESULTS

### *Effect of increasing doses of probenecid on the concentration of homovanillic acid in the striatum of the mouse*

The concentration of homovanillic acid in the striatum was estimated 1.5 hr after the administration of increasing doses of probenecid. Control animals were injected with

0.9% w/v sodium chloride solution. The dose-response curve obtained is given in Fig. 1. This shows that the maximum response to probenecid is obtained with a dose of 200 mg/kg.

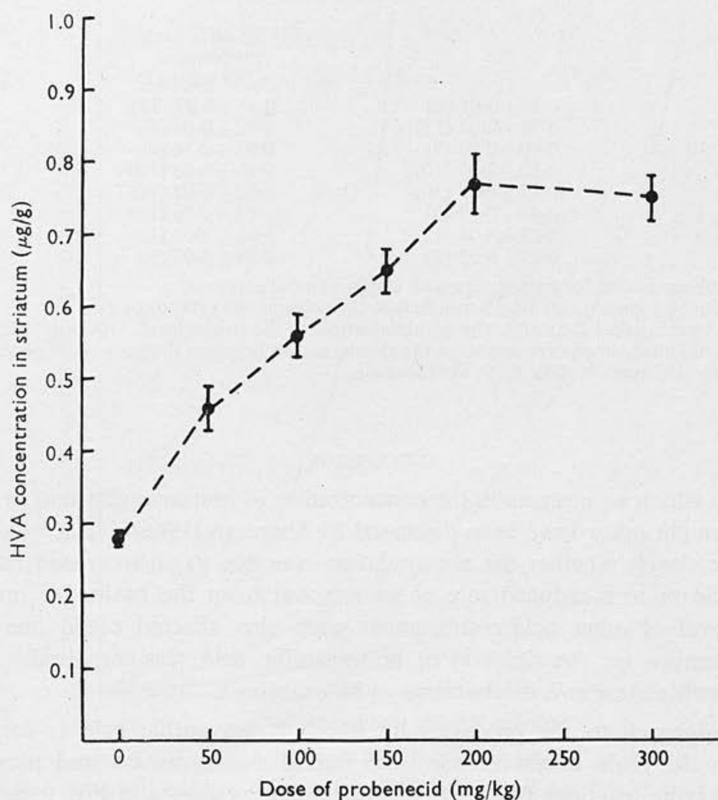



Fig. 1. Dose-response curve for the effect of probenecid on the concentration of homovanillic acid in the striatum of the mouse.

*Increase in the concentration of homovanillic acid in the striatum of the mouse produced by a standard dose of probenecid in combination with other drugs*

Several types of drugs can cause an elevation of the concentration of homovanillic acid in the striatum of the mouse (Sharman, 1966). The effects on the concentration of striatal homovanillic acid of some of these drugs given alone and in combination with a dose of probenecid (100 mg/kg) are shown in Table 1. The increase due to the administration of probenecid is larger, when this drug is given in combination with 2-aminotetralin (β-tetrahydronaphthylamine), 30 mg/kg; spiroperidol (8-[3-(4-fluoro-benzoyl)-propyl]-4-oxo-1-phenyl-1,3,8-triaza-spiro [4,5] decane), 0.5 mg/kg; chlorpromazine, 1 mg/kg and 5 mg/kg, and M99 (6,14-endoetheno-7-(2-hydroxy-2-pentyl)-tetrahydro-oripavine hydrochloride), than when given on its own. Spiroperidol (0.1 mg/kg) did not increase the effect of probenecid (100 mg/kg), although this dose of spiroperidol caused a significant increase in the concentration of homovanillic acid when given alone.

TABLE 1

THE EFFECT OF SOME DRUGS GIVEN ALONE AND IN COMBINATION WITH PROBENECID ON THE CONCENTRATION OF HOMOVANILLIC ACID IN THE STRIATUM OF THE MOUSE

| Drug (mg/kg) | Concentration of homovanillic acid in the striatum in μg/g tissue±s.e.m. | With additional probenecid (100 mg/kg) | Difference±s.d. |
|---|---|---|---|
| Control | 0·28±0·01 (9) | 0·56±0·03 (18) | 0·28±0·04 |
| Probenecid, 100 | 0·56±0·03 (18)† | 0·77±0·04 (9) | 0·21±0·05 |
| 2-Aminotetralin, 30 | 0·40±0·03 (9) † | 0·91±0·10 (9) | 0·51±0·11* |
| Spiroperidol, 0·1 | 0·60±0·03 (10)† | 0·91±0·05 (10) | 0·31±0·07 |
| Spiroperidol, 0·5 | 0·83±0·02 (10)† | 1·42±0·08 (10) | 0·59±0·08* |
| Chlorpromazine, 1 | 0·40±0·02 (9) † | 0·85±0·06 (10) | 0·45±0·06* |
| Chlorpromazine, 5 | 0·85±0·04 (11) † | 1·36±0·04 (11) | 0·51±0·05* |
| M99, 0·2 | 0·49±0·03 (9) † | 0·98±0·07 (9) | 0·49±0·07* |

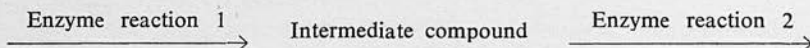
Number of observations for each estimation is given in parentheses.
Drugs in column 1 were given 10–15 min before the probenecid (100 mg/kg).
Estimations were made 1·5 hr after the administration of the probenecid, (100 mg/kg).
* 2×2 factorial analysis of variance shows that interaction between drugs is significant $P<0·05$.
† Significantly different $P<0·01$ from control value.

## DISCUSSION

The ways in which an increase in the concentration of homovanillic acid in the striatum of the mouse might occur have been discussed by Sharman (1966), but it was not possible to differentiate clearly whether the accumulation was due to an increased rate of formation of the acid or to a reduced rate of its removal from the brain, for only when the rates of removal of other acidic substances were also affected could one feel certain that the mechanism for the removal of homovanillic acid was impaired. The present experiments enable these two mechanisms to be examined.

A simple approach to the processes by which homovanillic acid is formed in and removed from the brain of the mouse is to regard the formation and removal as two consecutive enzyme reactions in an irreversible chain, as shown in Fig. 2, and to assume

Fig. 2.




that probenecid acts by competitive inhibition of the second enzyme. It can be shown (Webb, 1963) that, in such a situation, the concentration of the intermediate compound, in this case homovanillic acid, depends on the rate of its formation and on the ratio of the maximum rates of the two enzyme reactions. Figure 3 shows some theoretically calculated curves for the relationship between the concentration of such an intermediate compound and the rate of the first enzyme reaction and also the effect of changes in the ratio of the maximum rates of the two reactions. From such curves, in the absence of detailed information about conditions prevailing in the mouse striatum, it can be taken as a first approximation that, for low concentrations of the intermediate compound, a two-fold increase in the rate of the first reaction is reflected by a doubling of the concentration of the intermediate.

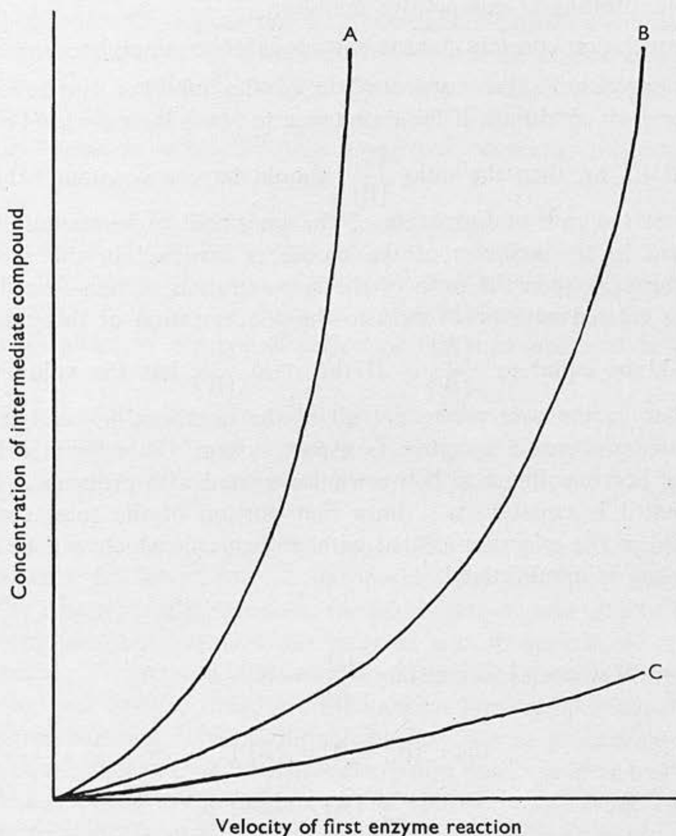



Fig. 3. Typical curves showing the relationship of the concentration of the intermediate compound between two consecutive enzyme reactions to the velocity of the first enzyme reaction.

$$\text{Curve A when } \frac{V_{max} \text{ reaction 1}}{V_{max} \text{ reaction 2}} = 2$$

$$\text{Curve B when } \frac{V_{max} \text{ reaction 1}}{V_{max} \text{ reaction 2}} = 1$$

$$\text{Curve C when } \frac{V_{max} \text{ reaction 1}}{V_{max} \text{ reaction 2}} = 0.5.$$

The increase in the concentration of the intermediate compound when the second enzyme is competitively inhibited can be described (Webb, 1963) by the equation

$$\frac{[B_i]}{[B]} = 1 + \frac{[I]}{K_i} \quad \ldots\ldots (1)$$

where $[B_i]$ = Concentration of intermediate after competitive inhibition of enzyme 2.
$[B]$ = Concentration of intermediate without inhibition of enzyme 2.

$[I]$ = Concentration of competitive inhibitor.

$K_i$ = Dissociation constant for the enzyme-inhibitor complex.

In the present experiments the concentration of the inhibitor, probenecid was kept constant. Under such conditions if the time taken to reach the new level of intermediate does not exceed 1.5 hr, then the ratio $\frac{[B_i]}{[B]}$ should have a constant value and should be independent of the rate of formation of the intermediate compound. If all of the homovanillic acid in the striatum of the mouse is involved in the system which is sensitive to probenecid, then the ratio of the concentration of homovanillic acid in the striatum of mice treated with probenecid to the concentration of this acid in mice not so treated should be equal to $\frac{[B_i]}{[B]}$. If the ratio $\frac{[B_i]}{[B]}$ has the value n then it can be calculated that, in the case where not all of the homovanillic acid in the striatum is involved in the probenecid sensitive transport system, the difference in the striatal concentrations of homovanillic acid between mice treated with probenecid and in similar mice not so treated is equal to n-1 times that portion of the total concentration of homovanillic acid in the mice not treated with probenecid, which will be affected when this inhibitory drug is administered.

i.e.:

From equation (1) when [I] is constant, $\frac{[I]}{K_i} = K$

$$\text{then, } [B_i] - [B] = [B]\,K \quad \ldots\ldots (2)$$

$$\text{let } \frac{[B_i]}{[B]} = n$$

then from equation (1) $\quad n = 1 + K$

substituting for K in equation (2) $\quad [B_i] - [B] = (n-1)\,[B]$

let [x] be a part of the initial homovanillic acid concentration unaffected by probenecid, then $[B] + [x]$ increases to $[B_i] + [x]$ after probenecid.

$$([B_i] + [x]) - ([B] + [x]) = [B_i] - [B] = (n-1)\,[B]$$

It is therefore possible to distinguish an increase in the concentration of homovanillic acid due to competitive inhibition of the active transport mechanism, or due to the formation of the acid at a site where this mechanism is not effective, from an increase due to an increased formation of homovanillic acid at the site where it is normally formed, or resulting from non-competitive inhibition of the second enzyme reaction. This latter effect is equivalent to reducing the maximum rate of the second enzyme and the result of such a change can be predicted from the curves given in Fig. 3.

Assuming that all of the homovanillic acid in the striatum of the normal mouse is removed by the probenecid sensitive transport system then, in the present experiments, the value of n calculated from the effect of probenecid in control animals is found to be

2 for a dose of 100 mg/kg. Thus:

1. If a drug increases the concentration of homovanillic acid by competitive inhibition of the active transport system, or by causing the formation of this acid at a site where this system is not effective, then the administration of probenecid (100 mg/kg) in combination with such a drug will produce an additional increase in the concentration of homovanillic acid equal to or smaller than the increase seen after this dose of probenecid given alone.

2. If a drug produces an increase in the concentration of homovanillic acid by an increase in the rate of formation of this acid, or by non-competitive inhibition of the active transport mechanism, then the administration of probenecid (100 mg/kg) in combination with such a drug will produce an additional increase in the concentration of homovanillic acid equal to the concentration of this acid observed in animals treated with the drug alone.

Furthermore, by subtracting the additional increase produced by probenecid (100 mg/kg) from the concentration of homovanillic acid observed in animals treated with the drug alone, it is possible to separate these two types of increase when they occur together.

The increases in the concentration of homovanillic acid seen after a multiple dose of probenecid and after the lower dose of spiroperidol (0.1 mg/kg) correspond with those proposed for the first type of mechanism, the additional increase due to probenecid (100 mg/kg) being approximately equal to the increase seen in the control animals after this dose of probenecid. After the administration of 2-amino-tetralin (30 mg/kg), chlorpromazine (1 mg/kg) and M99 (0.2 mg/kg), the increase has the magnitude required by the second type of mechanism. The additional increase due to probenecid (100 mg/kg) is approximately equal in each case to the concentration observed after treatment with each drug alone. The response to the larger doses of spiroperidol (0.5 mg/kg) and chlorpromazine appears to result from the action of the two types of mechanism. In these two cases the additional increase due to probenecid (100 mg/kg) is smaller than the concentration of homovanillic acid seen in animals treated with these drugs alone. This effect, particularly in the case of chlorpromazine, could be a result of the high rate of formation of homovanillic acid. The time taken to reach the new level of intermediate might be longer than 1.5 hr and thus the increase produced by probenecid would be smaller than expected from the equation.

Using the rate of depletion of dopamine from the mouse striatum after inhibiting the synthesis of this amine in the brain, Dickinson (personal communication) and Sharman (1966) have found evidence that chlorpromazine and spiroperidol cause an increased rate of utilization of dopamine, the amine from which homovanillic acid is formed, but in the case of spiroperidol, the increase in utilization was thought to be insufficient to account for the increase in homovanillic acid produced by this tranquillizing drug. The present results suggest that the action of low doses of spiroperidol in increasing the striatal concentration of homovanillic acid does not involve an increase in the rate of utilization of dopamine through its normal pathway.

An increase in the utilization of dopamine was not observed after treatment with M99 (Sharman, 1966). The possibility that this drug increases the homovanillic acid concentration by non-competitive inhibition of the active transport mechanism must therefore

be considered. The present results yield some indirect evidence that this is not the explanation for the effect of this drug on the concentration of homovanillic acid in the striatum. The dose-response curve relating the striatal concentration of homovanillic acid to the dose of probenecid shows that the concentration of homovanillic acid does not rise above 0.8 $\mu g/g$. This could represent the maximum concentration that it is possible to reach in 1.5 hr after total inhibition of the removal of the acid from the brain when the rate of formation of homovanillic acid is normal, and corresponds to a normal turnover rate of the acid of 0.05 $\mu g/g/min$.

Treatment with M99 together with probenecid elevated the homovanillic acid to 0.98 $\mu g/g$ in 1.5 hr, so that it would appear that at least part of the action of M99 is due to an increase in the rate of formation of homovanillic acid in the striatum.

## SUMMARY

1. The administration of probenecid, a competitive inhibitor of renal acid transport, to mice results in an increase in the concentration of 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid) in the striatum. This indicates that there is an active acid transport system in this tissue.

2. The effect on striatal homovanillic acid of giving a standard dose of probenecid alone and in combination with other drugs which change the concentration of this acid in the striatum was examined.

3. If the probenecid-sensitive acid transport system in the striatum is regarded as a simple monolinear enzyme chain, then it is possible to distinguish those drugs which increase the concentration of homovanillic acid by competitive inhibition of the acid transport system from those drugs which increase the rate of formation of the acid.

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# HOMOVANILLIC ACID AND DIHYDROXYPHENYLACETIC ACID IN THE STRIATUM OF MONKEYS WITH BRAIN LESIONS

D. F. SHARMAN,[1] L. J. POIRIER, G. F. MURPHY,[2] AND T. L. SOURKES

*Laboratory of Chemical Neurobiology, Allan Memorial Institute of Psychiatry*
*and*
*Laboratoire de Neuro-psychiatrie expérimentale, Faculté de Médecine, Université Laval, Qué.*

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Lesions were placed in the left ventromedial tegmental area of the brains of nine monkeys (*Macaca mulatta*). After 1–4 months, the brains were removed, and the striatum was dissected and prepared for chemical analysis. In seven animals in which the lesion was strictly unilateral, the striatal dopamine was very much reduced below the level found on the intact side of the brain. In the two monkeys in which the lesion impinged on the right side of the brain, the concentration of dopamine was reduced on both sides. Homovanillic acid and 3,4-dihydroxyphenylacetic acid, metabolic derivatives of dopamine, were measured in the left and right striata of four and five monkeys respectively. Both compounds were present at subnormal concentrations on the side of the lesion. The concentration of homovanillic acid did not fall to as great an extent as did that of dopamine. These results are discussed in relation to data reported by others in regard to (*a*) animals treated with tranquilizing drugs, and (*b*) brains of patients who died with Parkinson's disease.

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Neuf singes (*Macaca mulatta*), porteurs de lésions intéressant la région tegmentaire ventromédiane gauche du tronc cérébral, ont servi à cette étude. Les cerveaux furent prélevés après une période d'observation de 1 à 4 mois; les striata furent disséqués séparément en vue de déterminations chimiques. Chez sept des animaux porteurs d'une lésion strictement unilatérale la dopamine du striatum ipsilatéral était abaissée considérablement par comparaison avec le striatum contralatéral. Chex deux singes où la lesion a débordé du côté droit la dopamine striatale était abaissée des deux côtés. Chez quatre singes l'acide homovanillique et chez les cinq autres l'acide 3,4-dihydroxyphenylacétique furent mésurés au niveau des striata, droit et gauche. La concentration de ces deux dérivés métaboliques de la dopamine était au-dessous de la normale du côté de la lésion. Cependant la déplétion d'acide homovanillique n'était pas aussi accentuée que celle de la dopamine. Les résultats rapportés ici sont confrontés avec les données d'autres chercheurs obtenues chez (*a*) des animaux ayant reçu des tranquilisants; et (*b*) des cerveaux de patients ayant présenté un syndrome parkinsonnien.

Since the discovery of dopa and dopamine in the brain (1–3) and the relatively high concentration of the latter substance in the basal ganglia and certain associated structures (4, 5), there has been much interest in the neural functions of this amine. Much of our information about the metabolism of dopamine and its metabolites in the brain comes from pharmacological studies, but the most pertinent information at present comes from the direct determination of this catecholamine in necropsy material from patients who died with

[1]Medical Research Council Visiting Scientist. Permanent address: Institute of Animal Physiology, Agricultural Research Council, Babraham, Cambridge, England.
[2]Fellow of the Quebec Medical Research Council, 1965–1966, and graduate student, Department of Biochemistry, McGill University.

Parkinson's disease (6, 7) and from monkeys in which the placing of a specific unilateral lesion in the brain has resulted in chronic disturbances of posture, including hypokinesia and tremor of the contralateral limbs (8–10). In both cases, clinical and experimental, there is a distinct decline in the concentration of dopamine in the striatum. There may be a loss of striatal serotonin as well, and in the experimental condition changes in both amines have been correlated with specific postural deficits (9).

Many possibilities can be suggested to account for the decrease in dopamine in Parkinson's disease, and some of these have been tested. Thus, Bernheimer and Hornykiewicz (11) have already excluded from consideration a decrease in dopa decarboxylase, but a reduction in tyrosine hydroxylase activity, an earlier step in the biosynthesis of dopamine, might produce the same result. Another possibility, based upon histological and neurochemical evidence, is that the concentration of dopamine in the striatum is dependent upon the presence in that tissue of fibers originating in the substantia nigra which contain or release dopamine (8, 12). Lesions affecting the latter structure would then result in a loss of nigro–striatal fibers and the dopamine that they form. On this basis, the concentration of striatal dopamine would be proportional to the number of intact innervating fibers originating in the substantia nigra.

Bernheimer and Hornykiewicz (13) have measured the concentration of an important metabolite, homovanillic acid, in the brains of Parkinsonian patients. This substance was found, like dopamine, to be reduced in concentration in the caudate nucleus and putamen, but not as much as its precursor. It was therefore of interest to determine whether monkeys with characteristic brain lesions resulting in the loss of striatal dopamine would show changes in homovanillic acid. The availability of a new method for the estimation of dopac (3,4-dihydroxyphenylacetic acid) has permitted us to measure this substance as well.

## Experimental

Nine monkeys (*Macaca mulatta*) were used in this work. Electrolytic lesions were made unilaterally as previously described (8), and the animals were observed for 1–4 months before being killed. The brain was removed quickly and the striatum dissected and weighed. The tissue was rapidly frozen, and held in this state until ready to be extracted for determination of the metabolites. The remainder of the brain was reserved for histological study. This revealed that there was more or less severe cell loss in the substantia nigra on the left side in all animals. In addition, there was a slight loss of cells on the right side in monkey BP 25, and a large loss in BP 28. Details of the histological and postural deficits in these monkeys will be presented elsewhere.

The determination of dopamine in four monkeys of series X was performed by the method of Laverty and Sharman (14); for the five animals of series XI, the procedure of Murphy and Sourkes (15) was employed. Homovanillic acid was adsorbed from a deproteinized tissue extract on a column of Dowex-1

X-2 anion exchange resin (16), eluted with 0.1 *N* hydrochloric acid, and estimated fluorometrically by a modification of the method of Anden, Roos, and Werdinius (17). Dopac was determined, after adsorption on and elution from alumina, by an ethylenediamine reaction employed in the following way (Murphy and Sharman, unpublished). Aliquots of the eluates were treated with acetic anhydride (14), after which the reaction mixture was extracted with dichloromethane to remove acetylated catecholamines and neutral compounds. Acetylated dopac was extracted into another portion of the solvent after the reaction mixtures were rendered strongly acidic. The solvent was evaporated, and the residue condensed with ethylenediamine at 63° for 20–25 minutes. Dopac yields the typical yellow fluorophore of ethylenediamine conjugates but, following acidification beyond pH 3 and subsequent neutralization of the reaction mixture, a distinct blue fluorophore is obtained. Fluorescence at 450 mμ is recorded, with activation at 390 mμ (uncorrected instrumental values).

## Results

Analytical results for the monkeys are set out in Table I. In each animal the concentration of striatal dopamine in the operated side was lower than in the intact side of the brain. In some animals (BP 25, 26, and 27) the concentration in the unoperated side was somewhat lower than usually seen, and in one monkey (BP 28) in which the lesion impinged on the opposite (right) side and was associated with bilateral involvement of the nigra, the concentration was

TABLE I
Concentration of dopamine and other metabolites in striatum of monkeys*

| No. | Side | Dopamine (D) | Homovanillic acid (HVA) | HVA:D† | Dopac | Dopac:D† |
|---|---|---|---|---|---|---|
| Series X | | | | | | |
| 18 | Left | 2.4 | 6.5 | 2.28 | | |
| | Right | 7.2 | 11.2 | 1.31 | | |
| 22 | Left | 3.0 | 7.4 | 2.07 | | |
| | Right | 6.5 | 12.1 | 1.56 | | |
| 23 | Left | 0.4 | 4.7 | 9.86 | | |
| | Right | 4.7 | 9.6 | 1.72 | | |
| 30 | Left | 1.0 | 5.6 | 4.71 | | |
| | Right | 10.1 | 17.0 | 1.42 | | |
| Series XI | | | | | | |
| 7 | Left | 1.6 | | | <0.1 | <0.01 |
| | Right | 4.8 | | | 0.1 | <0.01 |
| 25 | Left | 0.1 | | | 0.3 | 4.6 |
| | Right | 2.4 | | | 0.3 | 0.1 |
| 26 | Left | 0.4 | | | 0.1 | 0.2 |
| | Right | 2.9 | | | 0.4 | 0.1 |
| 27 | Left | 1.6 | | | 0.4 | 0.2 |
| | Right | 3.3 | | | 0.7 | 0.2 |
| 28 | Left | <0.1 | | | 0.2 | 4.7 |
| | Right | 0.3 | | | 0.3 | 1.2 |

*Monkeys (our series BP) had a lesion administered to the ventromedial tegmental area of the left side. Concentrations are all expressed as micrograms per gram wet weight of tissue.
†Molar ratio, calculated before rounding the concentrations to the first decimal place.

very low. The interpretation of these changes will be dealt with elsewhere, in conjunction with the histological findings.

Substantial amounts of homovanillic acid were found in the striatum, ranging from 9.6 to 17.0 μg/g for the unoperated side. Less was present in the operated side. On a molar basis there was more of this compound than of dopamine. A comparison of the values for the two sides of the brain shows that the decrease in striatal homovanillic acid is proportionately smaller than the fall in dopamine. For example, in two monkeys (BP 23 and 30) the concentration of dopamine in the operated side was less than 10% of that in the unoperated side, but the homovanillic acid declined by only 51 and 67% respectively. If one considers the homovanillic acid to reflect the formation and metabolism of neuronal dopamine, then the molar ratios of these two substances, shown in Table I, indicate that when the number of dopamine-producing fibers in the striatum is reduced, the remaining ones are capable of increasing their production very substantially.

In contrast to the amounts of homovanillic acid, little dopac was present. In this series it never exceeded a concentration of 0.70 μg/g, indicating that dopac is a minor metabolite in this tissue. In four of five animals in which it was measured, there was less dopac in the striatum of the left (operated) side than in the right.

## Discussion

The concentration of homovanillic acid in the striatum of the monkeys in this work ranged from 4.7 to 17.0 μg/g. The lowest values (4.7–7.4 μg/g) were found in the operated side of the brain, where the concentration of dopamine was much reduced, as observed on previous occasions. Hence, these low values for homovanillic acid can be understood as arising from the deficient number of dopamine-forming, or "dopaminergic" fibers in the striatum (9, 10). The concentration of homovanillic acid in the unoperated side of the brain was 9.6–17.0 μg/g and was of the same order of magnitude as found in the caudate nucleus of dog and rabbit (18), but somewhat higher than reported for the caudate nucleus and putamen of man (13, 19) or the corpus striatum of rabbit (17).

The concentration of dopac was much smaller than that of homovanillic acid, and did not seem to bear any clear relation to the concentration of dopamine in the striatum. The mean value was about 0.3 μg/g, which is similar to that found by Anden *et al.* recently (20) (but not earlier (21)) for the corpus striatum of the rabbit, and very much smaller than the value given ($1.34 \pm 0.46$ μg/g) by Matsuoka *et al.* (22) for the caudate nucleus of the rabbit.

The difference in patterns may lie in the fact that homovanillic acid is a terminal product of the metabolism of dopamine, accumulating to a certain level before being lost from the nervous tissue, whereas dopac can undergo transformation to homovanillic acid; the concentration of dopac would then depend upon an additional factor, i.e. the relative activity of catechol *O*-methyl

transferase in its vicinity. Another possibility that would account for this difference is that most of the striatal dopamine may be readily methylated at the 3-hydroxy position, so determining homovanillic acid as the main terminal product; if only small amounts of dopamine gained access to monoamine oxidase, then only very small amounts of dopac could be formed.

In postmortem material from patients with Parkinson's disease, Bernheimer and Hornykiewicz found ratios of homovanillic acid to dopamine of about 1.8 for the caudate nucleus and 3.6 for the putamen. Corresponding values for the control brains were 1.2 and 1.1. In the present monkeys with brain lesions, the ratio was 2.1–9.9 for the operated side, compared with 1.3–1.7 for the unoperated side. As the latter side had concentrations of dopamine well within the normal range, it may be presumed that there was no metabolic abnormality on this side, and that the homovanillic acid concentrations observed represent "normal" values for the monkey striatum. Bernheimer and Hornykiewicz have expressed some concern over the validity of the data for homovanillic acid and dopamine obtained in postmortem tissues (13), especially because they have found experimentally that a fall in brain dopamine occurring after death may be accompanied by an increase in homovanillic acid. Their tissues were obtained only at some variable, but relatively long, period after the death of the patients, whereas in the present work the striatum was dissected and frozen within 30 minutes of the death of the monkey. Although chemical changes could have occurred in this time also, one would expect them to be smaller and more consistent. In any case, the similarity of the patterns for the experimentally controlled monkeys with brain lesions and the patients with Parkinson's disease is striking. The results can be explained on the basis of a reduced supply of dopaminergic fibers to the striatum from the substantia nigra (9) as a result of a lesion in that tissue, and leading to a diminution in the concentrations of dopamine, homovanillic acid, and dopac in the striatum. At the same time, there would be an increased rate of formation or release of dopamine (or both) by the remaining intact neurons, resulting in relatively more homovanillic acid. This increase might be initiated by a feedback mechanism working through some neural connection such as the striato–nigral pathways linking the caudate nucleus and putamen to the nigra. Such a compensatory mechanism has already been suggested by Roos (20, 23) in connection with the action of some major tranquilizers. Drugs like chlorpromazine and haloperidol, which can block the effects of dopamine, increase the concentration of homovanillic acid and dopac in the corpus striatum, without affecting the level of dopamine, serotonin, or 5-hydroxyindoleacetic acid.

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# RELEASE BY TUBOCURARINE OF DOPAMINE AND HOMOVANILLIC ACID FROM THE SUPERFUSED CAUDATE NUCLEUS

BY P. J. PORTIG, D. F. SHARMAN AND MARTHE VOGT
*From the Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge*

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## SUMMARY

1. The release of dopamine and its metabolite homovanillic acid from the 'superfused' caudate nucleus was studied in the cat. Fluorimetric assay of the two compounds was carried out on 30 min samples of artificial cerebrospinal fluid perfused through the anterior horn of one lateral ventricle.

2. When tubocurarine 0·5 or $1 \times 10^{-4}$ g/ml. was added to the perfusion fluid, amounts of dopamine of the order of 0·1 ng/min, and very much larger quantities of homovanillic acid (up to 8 ng/min) appeared in the perfusing fluid.

3. No release of dopamine was obtained under conditions when tubocurarine failed to produce any clinical signs or when gallamine was substituted for tubocurarine.

## INTRODUCTION

In experiments which are still in progress we are investigating whether stimulation, by a variety of means, of the cat's caudate nucleus, releases substances which might be transmitters or their metabolites. In devising a method of demonstrating the release of such substances, we were particularly anxious to avoid any lesion to the caudate nucleus itself, and therefore chose the perfusion of the anterior horn of the lateral ventricle (Carmichael, Feldberg & Fleischhauer, 1964) as a safe technique which allows testing of the perfusate for substances given off from the surface of the 'superfused' caudate nucleus. When the *substantia nigra* was stimulated electrically in such perfusions, dopamine was found to be frequently, but not consistently, released into the perfusion fluid; the metabolite of dopamine, homovanillic acid, was released with more regularity. There were, however, occasions when dopamine appeared in the perfusion fluid when no external stimuli were applied, and such events

seemed to be correlated with shivering or slight trembling of the musculature of limbs or trunk. It therefore seemed desirable to produce shivering intentionally and to study its effect on the release of dopamine and homovanillic acid. Shivering was induced by perfusing the cerebral ventricles with a solution of tubocurarine (Carmichael, Feldberg & Fleischhauer, 1962). Some of the results have been reported to the Physiological Society (Portig & Vogt, 1967).

## METHODS

*Collection of ventricular perfusates.* Cats of either sex and weighing at least 2·5 kg were anaesthetized with ether followed by chloralose (60–80 mg/kg intravenously) and the heads fixed in a stereotaxic instrument for superfusion of the caudate nucleus. Following the description by Carmichael *et al.* (1964), the aqueduct was cannulated, and a 'Collison cannula', to be used as an outflow, was screwed into the left side of the skull at the Horsley Clarke co-ordinates A 11 and L 4·5. When contact with the ventricle had been established, a steel needle connected to a slow-infusion pump and filled with artificial cerebrospinal fluid was lowered into the anterior end of the left anterior horn to co-ordinates A 18–19, L 2 and H 5.

The tubing in the aqueduct was then occluded in order to divert the perfusion fluid into a collecting tube inserted into the Collison cannula. This tube ended about 9 cm below the opening of the inflow needle. Rate of delivery from the pump was about 4·5 ml./hr. Any slowing of the flow indicating obstruction and leading to an increased intracranial pressure had to be corrected immediately by adjusting the position of the Collison cannula, since it was found that a rise in intracranial pressure invariably led to a fall in the concentration of various substances released into the perfusate. The perfusion fluid had the composition (g/l.) NaCl 8·1, KCl 0·25, $CaCl_2$ 0·14, $MgCl_2$ 0·11, $NaHCO_3$ 0·3, $Na_2HPO_4$ 0·07, $CO(NH_2)_2$ 0·13, glucose 0·6. Ascorbic acid 0·01 was added whenever dopamine was to be estimated. (+)-Tubo-curarine chloride (Koch-Light) or gallamine triethiodide (Flaxedil, May & Baker) were dissolved in the perfusion fluid to make up concentrations of either 1:10000 or 1:20000 and perfusion was switched over to these mixtures as soon as a sufficient number of control samples had been obtained in the absence of the drug. The effluent was collected in graduated tubes standing in ice water; these tubes contained 2 mg ascorbic acid for all dopamine estimations. All samples were frozen and kept at −17° C if not analysed immediately. When dopamine was to be measured, the samples were acidified with 0·1 N-HCl before freezing.

Blood pressure was recorded from a femoral artery by pressure transducer, an electrode (bipolar, coaxial type, external diameter 0·45 mm) was lowered into the right caudate nucleus to record spontaneous or evoked potentials on a cathode ray oscilloscope, and the electroencephalogram was recorded with the aid of a steel pin from the right secondary visual area on a Grass polygraph. A Starling pump was used for artificial respiration.

*Chemical procedures.* Dopamine was estimated by the method of Laverty & Sharman (1965) slightly modified as follows: approximately 2 ml. of acidified perfusate was made up to 5 ml. with 0·01 N-HCl and acetylated by adding 0·5 ml. acetic anhydride and, gradually, with stirring, 1 g of solid $NaHCO_3$. Acetylation was complete in about 15 min. The mixture was extracted with 10 ml. dichloromethane and spun if necessary; the bottom layer was reduced to a small volume by a stream of air, applied to alkali-washed Whatman No. 50 paper and chromatographed in a mixture of toluene:ethylacetate:methanol:water 10:4:5:5 for about 3 hr at 26° C. Determination of the position of dopamine reference spots, elution, condensation with ethylene diamine and fluorimetry in a filter fluorimeter were carried out as described by Laverty & Sharman (1965). Recovery was checked each time and ranged from 70 to 80 %. A few ng of dopamine can be detected by this method.

Homovanillic acid extraction was simplified by modifying an earlier method (Sharman, 1963) and avoiding the chromatographic step. For the development of fluorescence ferricyanide and ammonia were used (Andén, Roos & Werdinius, 1963), and the fluorescence was measured with an Aminco-Bowman spectro-photo-fluorimeter. An interference filter (Grubb Parsons Filter Type G.P., peak wave-length 4262 Å and 44 % transmission) was placed into the path of the emitted light, and this, by reducing unspecific fluorescence, increased the sensitivity several-fold.

The details were as follows: To each sample of perfusate 0·2 ml. conc. $HClO_4$ followed by 0·3 g KCl were added, the mixture was spun and the deposit discarded. Concentrated HCl (0·1 ml.) was added to the supernatant and followed by enough solid NaCl to saturate the mixture, which was then extracted by shaking for 5 min with 10 ml. *n*-butyl acetate, the solvent used by Giacalone & Valzelli (1966) to extract 5-hydroxyindolyl acetic acid. (The butyl acetate was purified by distillation and saturated with water before use.) After centrifuging, the butyl acetate was pipetted off into a stoppered tube which contained 2 ml. of 0·05 M solution of Tris (2-amino-2-(hydroxymethyl) propane-1,3-diol). By shaking for 5 min the homovanillic acid was back-extracted into the Tris. The mixture was spun, the butyl acetate layer discarded and the watery layer divided into three portions, one of which was used as the unknown, the second as an internal standard and the third as a blank. The method allows estimation of total amounts down to 50 ng, and uses 100 ng homovanillic acid as the 'internal standard'. Recovery averaged 75 %. The addition of $HClO_4$ before the extraction with butyl acetate can be omitted, but the omission may lead to higher blanks.

Whenever drugs were added to the perfusion fluid, careful checks were made that neither 'blanks' nor 'internal standards' were modified by their presence. No difficulties were encountered with either tubocurarine or gallamine.

## RESULTS

When the anterior horn of one lateral ventricle was perfused with tubocurarine 1:20000 for 2½ hr or longer, dopamine appeared in the perfusate in small, somewhat variable, but measurable, concentrations (Fig. 1). Release persisted for periods up to 2½ hr. Perfusions led, with some delay, to twitches, salivation, shivering and large deflexions on the e.e.g. registered from one secondary visual area. As soon as twitches were detected, they were stopped by the intravenous injection of gallamine (6–12 mg of the triethiodide per dose), to avoid possible mechanical artifacts. Some experiments were also performed during continuous intravenous infusion of gallamine so that movements were avoided altogether.

Carmichael *et al.* (1962) have shown that tremor is induced by an action of (+)-tubocurarine on the hypothalamus. In one experiment, therefore, the (+)-tubocurarine was delivered to the third and not to the lateral ventricle by connecting a second infusion pump to the tubing inserted into the aqueduct, and collecting from the Collison cannula the combined perfusates of lateral and third ventricles. Dopamine appeared in the perfusion fluid within the first half hour of tubocurarine infusion, but the quantity was not clearly greater than when only the anterior horn of the

lateral ventricle was perfused, though release was somewhat more prolonged (Fig. 1*c*).

The remaining experiments on the release of dopamine were carried out with shorter periods of infusion of tubocurarine with the following objects in mind: to see whether the effect was repeatable, whether it was correlated with the pharmacological effects elicited by tubocurarine, and whether it was specific for this drug. Since successful perfusions without gradual deterioration in flow can rarely be carried out for more than

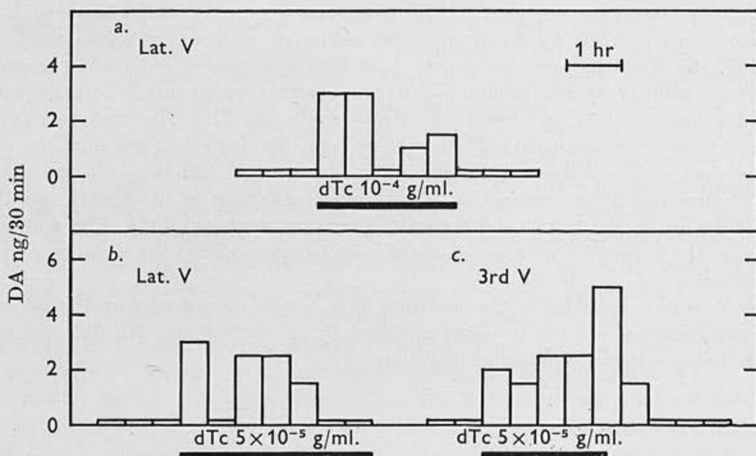


Fig. 1. Release of dopamine by prolonged cerebroventricular perfusions with tubocurarine (dTc).

Dopamine (DA) determined in 30 min samples of artificial cerebrospinal fluid; dTc perfused through the anterior horn of a lateral ventricle (*a* and *b*), or through the 3rd ventricle (*c*), of a cat. The columns represent concentrations in consecutive samples, expressed as ng/30 min, and not corrected for recovery. Heavy bars: perfusion of ventricle with tubocurarine $10^{-4}$ g/ml. (*a*), or $5\times10^{-5}$ g/ml. (*b* and *c*).

about 6 hr, tubocurarine was given for 1 hr at a time, thus allowing for a suitable number of control periods before and after the two infusions. With these shorter perfusions, the amount of dopamine released was occasionally hardly above the threshold of the method. However, when the amount was measurable, two periods of tubocurarine usually produced two rises of similar size and time course. The one exception suggests that release of dopamine is linked to the presence of clinical signs: in this experiment, dopamine did not appear during the first, but appeared during the second administration of tubocurarine; visible effects (twitches and large deflexions in the electroencephalogram) were present only on the second occasion (Fig. 2, *a* and *b*).

The specificity was tested in two experiments, in which the effect of

perfusing tubocurarine was compared with that of perfusing gallamine, both drugs being used in a concentration of $10^{-4}$ g/ml. Figure 2 (*c* and *d*) illustrates one of them, and shows that release occurred only during the administration of tubocurarine. When the drugs were given in a reverse order in the second experiment, the result was the same.

Fig. 2. Release of dopamine by short perfusions of a cerebral ventricle with tubocurarine (dTc).

Dopamine (DA) determined in 30 min samples of artificial cerebrospinal fluid perfused through the anterior horn of a lateral ventricle of the cat. The columns represent concentrations in consecutive samples, expressed as ng/30 min, and not corrected for recovery. Heavy bars: perfusion of ventricle with tubocurarine $5 \times 10^{-5}$ g/ml. (*a* and *b*), $10^{-4}$ g/ml. (*c*), and gallamine $10^{-4}$ g/ml. (*d*).

Whenever dopamine is released in the brain, it is subject to the action of the enzymes monoamine oxidase and O-methyl transferase and transformed into homovanillic acid. Any dopamine appearing in the ventricular perfusate was thus just the 'over-spill' of what escaped such destruction. The determination of the metabolite should therefore give a truer picture of any release of dopamine and yield larger amounts of material. However, the methods for fluorimetric estimation of homovanillic acid are very much less sensitive than those available for dopamine, and it was not clear whether the increase in sensitivity obtained by the modifications

described under 'Methods' were sufficient for our purpose. Figure 3 shows that it was. The two experiments illustrate that perfusates collected during control periods contain measurable amounts of homovanillic acid, whereas the concentration of dopamine in such samples had usually been below the threshold of the method. In the experiment shown in Fig. 3*a*,

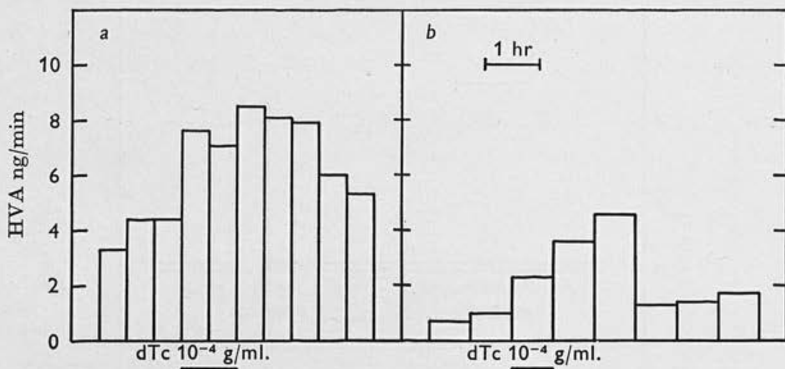


Fig. 3. Release of homovanillic acid by cerebroventricular perfusion with tubocurarine (dTc).

Homovanillic acid (HVA) determined in 30 min (*a*) or 45 min (*b*) samples of artificial cerebrospinal fluid perfused through the left anterior horn of one lateral ventricle of a cat. The columns represent concentrations in consecutive samples, expressed as ng/min, and corrected for recovery. Heavy bars: perfusion of ventricle with tubocurarine $10^{-4}$ g/ml.

tubocurarine was infused over a period of 1 hr; the concentration of homovanillic acid rose to approximately twice the resting concentration, stayed there for $2\frac{1}{2}$ hr, and then began to fall. In the second experiment (Fig. 3*b*), infusion lasted only 45 min, and the rise in homovanillic acid elicited by tubocurarine persisted for $2\frac{1}{4}$ hr.

There were two main differences between the pattern of release of dopamine and homovanillic acid. The first concerned the absolute values; for dopamine these hardly exceeded 4 ng (5 ng if corrected for recovery) in a collection period of half an hour, whereas for homovanillic acid they reached 240 ng (corrected). The second was the duration of the accelerated release which greatly outlasted the period of infusion for homovanillic acid but not for dopamine.

## DISCUSSION

The experiments have shown that perfusion of the cerebral ventricles with (+)-tubocurarine releases dopamine and its metabolite homovanillic acid into the fluid bathing the caudate nucleus. Since the caudate nucleus is the only dopamine-containing structure bordering on the anterior horn

of the lateral ventricle perfused in these experiments, it is clear that this nucleus must be the source of the amine. It is not at all certain, however, and not even likely, that the (+)-tubocurarine exerted its action directly on the caudate nucleus, since some of the drug diffuses to other parts of the ventricular system during prolonged perfusion. Several observations implicate the shivering and tremor elicited by intraventricularly administered tubocurarine in this release of dopamine: another muscle relaxant, gallamine, lacked both the capacity to release dopamine and to produce shivering (or any other clinical signs), and tubocurarine itself failed to release dopamine when shivering or tremor failed to appear. These findings suggest, though they do not prove, that 'spontaneous' release of dopamine in experiments in which there was spontaneous shivering was due to the shivering. Carmichael *et al.* (1962) have shown that the hypothalamus is the site to which tubocurarine must penetrate if tremor or shivering is to ensue; the activation of the caudate nucleus could thus be brought about by a reflex originating in the hypothalamus. Since, however, tubocurarine has many other motor and autonomic actions, it is quite possible that activation of the caudate nucleus could have taken place through other pathways.

Dopamine release lasted approximately as long as tubocurarine infusion, whereas release of homovallic acid outlasted the infusion for a considerable period. This observation is in keeping with observations made on the release of homovanillic acid by various stimuli applied for a few minutes only: the appearance of increased amounts of homovanillic acid rarely lasted less than 60 min (J. Portig & M. Vogt, to be published). The explanation appears to be the distance the metabolite has to travel if the cells activated in the caudate nucleus are situated far away from the ventricular surface. The same does not hold for dopamine, since most of the amine would be destroyed if it had to diffuse through a thick layer of tissue.

It would be of the greatest interest to know whether, and in what direction, the release of dopamine modifies, or contributes to, the motor response to tubocurarine infusion. The fact that tubocurarine-induced tremor is suppressed by intraventricular injection of large doses of dopamine (Carmichael *et al.* 1962) may or may not be relevant to the events within the caudate nucleus.

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# The effect of tropolone on the formation of 3,4-dihydroxyphenylacetic acid and 4-hydroxy-3-methoxyphenylacetic acid in the brain of the mouse

G. F. MURPHY, D. ROBINSON* AND D. F. SHARMAN*

*Laboratory of Chemical Neurobiology, Allan Memorial Institute of Psychiatry, McGill University, Montreal, Canada, and *Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge*

1. The development of a very sensitive and specific fluorimetric assay for 3,4-dihydroxyphenylacetic acid has made it possible to measure how inhibitors of the enzyme catechol-O-methyl transferase affect the relative concentrations of this acid and its O-methylated derivative 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid) in the brains of mice treated with L-3,4-dihydroxyphenylalanine or probenecid.

2. It was found that tropolone and tropolone-4-acetamide reduce the concentration of homovanillic acid in the brains of the treated mice to an extent dependent on the dose.

3. The concentration of 3,4-dihydroxyphenylacetic acid in the brain was increased by the administration of tropolone or tropolone-4-acetamide but the dose and response were not simply related to one another.

4. The results suggest that, *in vivo*, the formation of 3,4-dihydroxyphenylacetic acid is not always a simple alternative to the formation of homovanillic acid when the enzyme catechol-O-methyl transferase is inhibited.

The ability of some compounds to inhibit the enzyme catechol-O-methyl transferase (COMT) in the central nervous system *in vivo* has been studied by Carlsson, Lindqvist, Fila-Hromadko & Corrodi (1962), and by Carlsson, Corrodi & Waldeck (1963), who found that such compounds cause a fall in the concentration of 4-hydroxy-3-methoxyphenylethylamine (methoxytyramine) in the brains of mice treated with L-3,4-dihydroxyphenylalanine (L-DOPA) and a monoamine oxidase inhibitor. The activity of the enzyme in the brains of living mice has also been measured by Ross & Haljasmaa (1964a,b) with a radioisotopic method.

The development of a very sensitive specific fluorimetric assay for 3,4-dihydroxyphenylacetic acid (Sharman, Poirier, Murphy & Sourkes, 1967) has enabled us to study the ability of drugs to inhibit catechol-O-methyl transferase in the central nervous system. To do this we have observed the effects of such drugs on the relative concentrations of 3,4-dihydroxyphenylacetic acid and its methylated derivative homovanillic acid, in the brains of mice treated with L-DOPA or probenecid.

**Methods**

The following chemicals and reagents were used: homovanillic acid (Calbiochem); 3,4-dihydroxyphenylacetic acid (L. Light & Co. Ltd.; recrystallized from benzene); L-3,4-dihydroxyphenylalanine (L-DOPA; Calbiochem); Tris (hydroxymethyl) aminomethane (Tris; L. Light & Co. Ltd.); L-cysteine hydrochloride (Hopkin & Williams Ltd.; recrystallized from ethyl alcohol); Tropolone (2-hydroxy-2:4:6-cycloheptatriene-1-one; Aldrich Chemical Co. Inc.; recrystallized from light petroleum); Tropolone-4-acetamide (A. B. Hässle; recrystallized from dilute acetic acid); 1,2-diaminoethane distilled three times and kept at 4° C; *n*-butyl acetate distilled once and washed once with water. All other reagents and chemicals were of analytical reagent quality except for hydrochloric acid which was of micro-analytical reagent quality. Glass distilled water was used throughout.

Female albino mice weighing 15–35 g were used. Drugs were injected intraperitoneally, dissolved in 0·9% (w/v) sodium chloride solution, except for L-DOPA which was dissolved in sterile distilled water.

*Dissection and extraction of tissues*

The mice were stunned and then decapitated. The brain was rapidly removed and placed on a glass plate in an ice bath. In the experiments in which DOPA was injected, the brain stem was divided at the posterior border of the hypothalamus and all of the tissue rostral to the section, but excluding the olfactory lobes, was extracted. The tissue from one mouse was sufficient for the analysis of both dihydroxyphenylacetic acid and homovanillic acid. In the investigations on normal mice, the striatal region was dissected out (Sharman, 1966) and the tissue from three mice combined for the estimation of both the acids. The tissue samples were homogenized in 2 ml. of ice cold 0·1 N hydrochloric acid in a cooled, all glass homogenizer. The homogenate was transferred to a polypropylene or cellulose nitrate centrifuge tube (7 ml. capacity) and the homogenizer washed twice with 1 ml. of ice cold distilled water, the washings being combined and mixed with the homogenate. The centrifuge tube was then dipped into liquid nitrogen so that the homogenate was frozen. The frozen homogenate was placed in a refrigerator at 4° C until all the tissue samples to be extracted had been homogenized. The homogenates were then allowed to thaw at room temperature and concentrated perchloric acid (0·12 ml.; specific gravity, 1·72) was added to and mixed with each sample. Solid potassium chloride was then added in an amount just in excess of that required to saturate the homogenate, which was then thoroughly mixed. This procedure removes the perchlorate ion as insoluble potassium perchlorate while leaving the solution acid. The procedure is endothermic and this helps to keep the homogenate cold, also ensuring a good precipitation of the potassium perchlorate. The homogenate was then centrifuged at 0° C for 5 min with a radial acceleration at the tip of the tube of 147,000 m sec$^{-2}$ (15,000 *g*). The clear supernatant was transfered to a glass stoppered test tube (15 ml. capacity) and a few crystals of potassium chloride were added to ensure that the solution was saturated. *n*-Butyl acetate (10 ml.) was added and the tube shaken by hand for 5 min. The tube was centrifuged at room temperature for 1 min to separate the two layers and two portions (4·5 ml. each) of the *n*-butyl acetate extract were taken. One portion was added to 2 ml. of Tris solution (6 g/l. in distilled water) contained in a glass stoppered test tube (10 or 15 ml. capacity). The other was added to a 2·2 ml. portion of a mixture of 35 ml. water,

1 ml. 2 N hydrochloric acid and 1·5 ml. of 1,2-diaminoethane, also in a glass stoppered test tube. The tubes were cooled in ice, shaken for 3 min and then centrifuged for 1 min. The butyl acetate layers were discarded. Homovanillic acid was estimated in the Tris extract and dihydroxyphenylacetic acid in the 1,2-diaminoethane extract.

*Estimation of homovanillic acid*

Homovanillic acid was estimated fluorimetrically essentially as described by Andén, Roos & Werdinius (1963). Three portions (0·6 ml. each) of the Tris extract were taken. To one of them was added a known amount of homovanillic acid (usually 0·1 μg) and to another 0·2 ml. of a freshly prepared, solution of cysteine (1 mg/ml.). The latter tube served as a blank and the former enabled the development of the fluorescence to be checked and gave a standard for the calculation of the amount of homovanillic acid present. Potassium ferricyanide (1 ml. of 20 mg/l. in 5 N ammonium hydroxide) was then added to each tube and mixed. After exactly 4 min, 0·2 ml. of the cysteine solution was added to the two tubes which contained no cysteine and the contents of all tubes were mixed thoroughly. The fluorescence of the solutions was then measured in an Aminco-Bowman spectrophotofluorometer. The activating light wavelength was 315 mμ and the fluorescence light wavelength was 430 mμ. An interference filter with maximum transmission at 426·2 mμ and a band width of 9·9 mμ and 44% transmission (Grubb Parsons type G.P.) was placed in the fluorescent light path. Slits 5 mm wide were placed, immediately adjacent to the cuvette, in both the activating and fluorescent light paths. This arrangement yielded the smallest blank readings in comparison with the fluorescence developed from homovanillic acid.

*Estimation of 3,4-dihydroxyphenylacetic acid*

Two samples (1·0 ml. each) of the 1,2-diaminoethane extract were taken. To one of them was added a known amount of authentic 3,4-dihydroxyphenylacetic acid (usually 0·1 μg in a volume of 0·01 ml.). The solutions were then heated in a water bath at 60° C for 20 min in the dark. At the end of this time the tubes were cooled in an ice bath and hydrochloric acid (0·3 ml. of a 1 : 1 v/v dilution of concentrated hydrochloric acid (36% w/v) in distilled water) was added and mixed. The tubes were left in the ice bath for 10 min and neutralized by the addition of 1,2-diaminoethane (0·3 ml. of a 1 : 9 v/v dilution in distilled water). The fluorescence of the solution was immediately measured in an Aminco-Bowman spectrophotofluorometer. The activating light wavelength was 385 mμ and the fluorescence light wavelength was 450 mμ (uncorrected for any errors in the instrument). A Corning 3389 glass filter was placed in the fluorescence light path.

*Recovery experiments*

The recovery of authentic homovanillic acid and 3,4-dihydroxyphenylacetic acid was tested by adding amounts ranging from 0·1 μg to 2 μg to tissue homogenates and extraction and estimation as described above. For homovanillic acid the mean recovery was 70% ± 2·3% (S.E.M.), in eighteen tests, and for 3,4-dihydroxyphenylacetic acid the mean recovery was 65% ± 1·6% (S.E.M.), in seventeen tests.

**Results**

*Notes on the fluorimetric assay of 3,4-dihydroxyphenylacetic acid*

The present method for the estimation of this acid is an improvement on previous methods in two respects. First, the fluorescence intensity of the derivative is higher and second, the reaction is more specific for 3,4-dihydroxyphenylacetic acid.

The changes in the fluorescence characteristics are illustrated in Fig. 1. The yellow fluorescence (maximum wavelength 540 m$\mu$) which develops when 3,4-dihydroxyphenylacetic acid is condensed with 1,2-diaminoethane (Valk & Price, 1956) disappears on acidification and is replaced by a green fluorescence of lower intensity at a shorter wavelength (490 m$\mu$ when activated at 395 m$\mu$). As the solution is neutralized with ethylene diamine, this latter fluorescence at first increases in intensity, and then a second, more intense, blue fluorescence suddenly appears (maximum at 450 m$\mu$ ; wavelength of maximum activation, 385 m$\mu$). As more of the base is added, the fluorescence at 490 m$\mu$ gradually decreases and eventually disappears, but the intensity of the fluorescence at 450 m$\mu$ is only slightly increased. The conditions selected were those which, in model experiments, were found to give reproduceable results.

The fluorescence at 450 m$\mu$ appears to be specific for 3,4-dihydroxyphenylacetic acid and some closely related compounds which have an acid function. For example, if the reaction is carried out on 3,4-dihydroxyphenylalanine (DOPA) or $\alpha$-methyl-3,4-dihydroxyphenylalanine ($\alpha$-methyl-DOPA) very little fluorescence is developed.

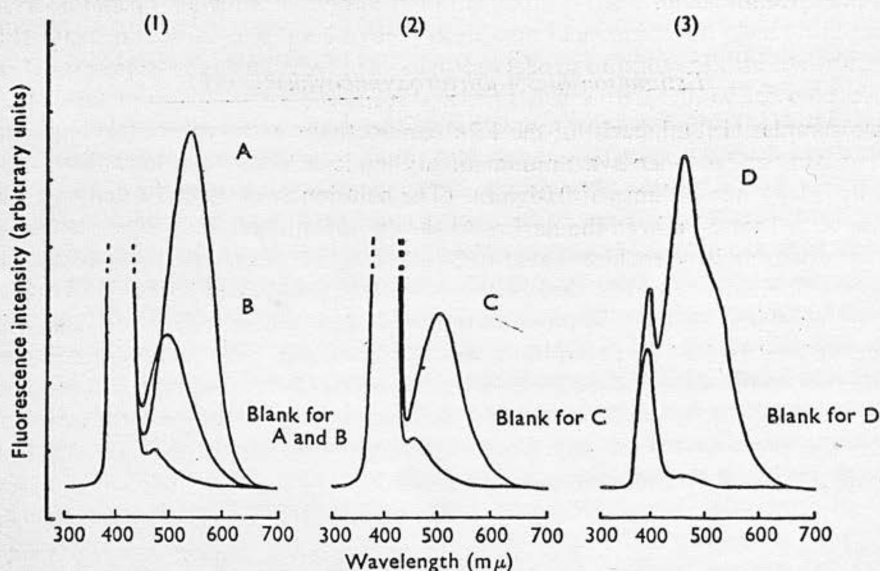


FIG. 1. Characteristics of the fluorescence derived from 3,4-dihydroxyphenylacetic acid. The reaction was carried out as described in the text using 0·2 $\mu$g of the acid. Where necessary the volume of the sample was adjusted to 1·6 ml. with water for each scan. (1) The characteristics of the fluorescence derived from 3,4-dihydroxyphenylacetic acid when activated at 405 m$\mu$. (A) After condensation with 1,2-diaminoethane ; (B) After the addition of hydrochloric acid. (2) Activation wavelength 385 m$\mu$. (C) As 1 (B). (3) Activation wavelength 385 m$\mu$. (D) After the addition of 1,2-diaminoethane solution. The scans in (3) were recorded at one tenth of the amplification used for 1 and 2.

If these two amino-acids are first acetylated to give the O,O,N-triacetyl derivatives, converting the amino group to a neutral amide group, then a blue fluorescence develops. 3,4-Dihydroxymandelic acid yields only a very small amount of fluorescence so that this acid will not interfere with the assay of 3,4-dihydroxyphenylacetic acid.

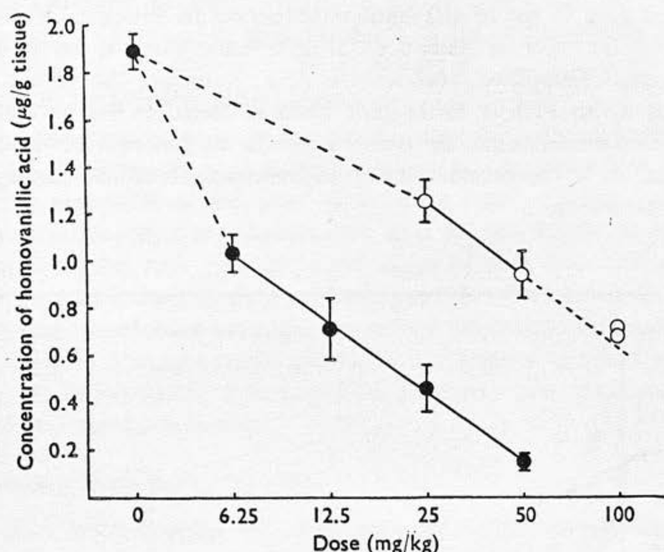


FIG. 2. Effect of tropolone and tropolone-4-acetamide on the concentration (±S.E.M.) of homovanillic acid on the brains of mice treated with L-DOPA. ●——●, Mice treated with L-DOPA (100 mg/kg) and tropolone for 1 hr; ○——○, mice treated with L-DOPA (100 mg/kg) and tropolone-4-acetamide for 1 hr.

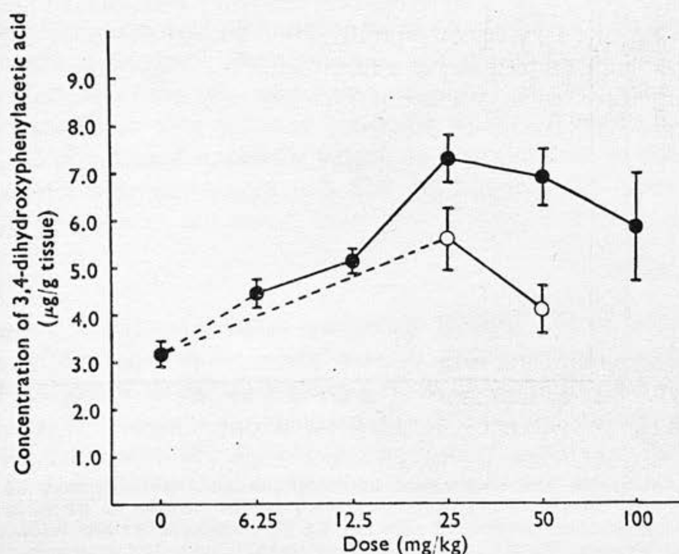


FIG. 3. Effect of tropolone and tropolone-4-acetamide on the concentration (±S.E.M.) of 3,4-dihydroxyphenylacetic acid in the brains of mice treated with L-DOPA. ●——●, Mice treated with L-DOPA (100 mg/kg) and tropolone for 1 hr. ○——○, mice treated with L-DOPA (100 mg/kg) and tropolone-4-acetamide for 1 hr.

The method, in common with many other fluorimetric methods, suffers from the difficulty of determining accurately the blank fluorescence. The fluorescence derived from extracts of cerebral cortex of rabbits and mice was slightly higher than that derived from the reagents alone. This increased fluorescence was found to be equivalent to 3,4-dihydroxyphenylacetic acid in a concentration of 0·03 μg/g tissue when approximately 0·5 g of brain tissue was extracted. In the present experiments the reagent blank has been used in the calculation of the concentration of 3,4-dihydroxyphenylacetic acid in the tissues from experiments in which L-DOPA was administered, because the error introduced by doing this is negligible. In the estimation of the endogenous 3,4-dihydroxyphenylacetic acid, however, the values might be over-estimated by about 10% if the reagent blank is used. It has not been proved that the fluorescence derived from the cortex is due to the presence of 3,4-dihydroxyphenylacetic acid, so a correction for the fluorescence from the tissue has been applied to these estimates.

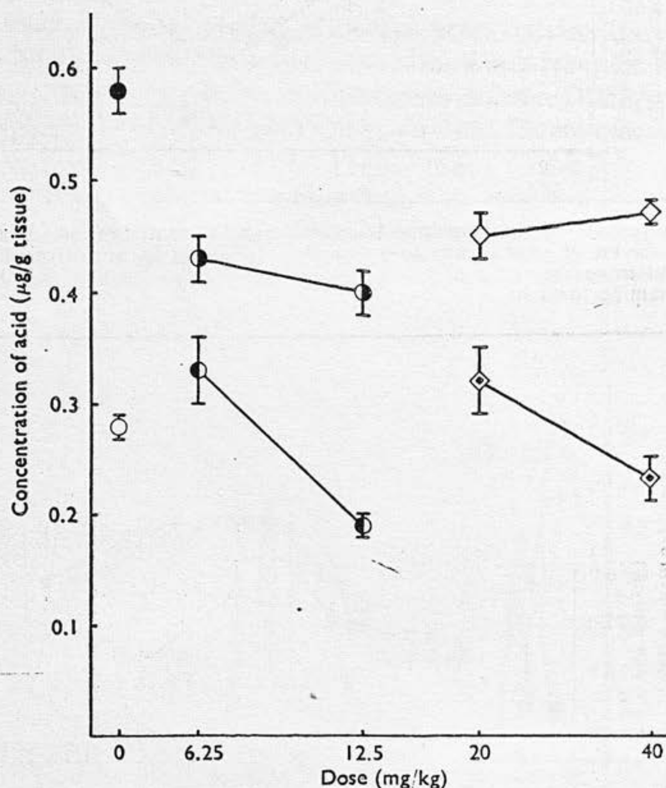


FIG. 4. Effect of tropolone and tropolone-4-acetamide on the concentrations (±S.E.M.) of homovanillic acid and 3,4-dihydroxyphenylacetic acid in the striata of mice treated with probenecid. All mice received probenecid (200 mg/kg intraperitoneally) and were killed after 1·5 hr. ●, Control homovanillic acid; ◐——◐, homovanillic acid after tropolone; ◈——◈, homovanillic acid after tropolone-4-acetamide; ○, control 3,4-dihydroxyphenylacetic acid; ◑——◑, 3,4-dihydroxyphenylacetic acid after tropolone; ◇——◇, 3,4-dihydroxyphenylacetic acid after tropolone-4-acetamide. The two regression lines for the fall in the concentration of homovanillic acid with the two drugs do not deviate significantly from parallel.

*Effect of tropolone and tropolone-4-acetamide on the concentration of homovanillic acid and 3,4-dihydroxyphenylacetic acid in the mouse brain*

*After treatment with L-DOPA*

Two groups of mice were injected with L-DOPA (100 mg/kg intraperitoneally), followed immediately by an intraperitoneal injection of tropolone or tropolone-4-acetamide in the test group. One hour later the mice were killed, the brains removed and analysed for their content of homovanillic acid and 3,4-dihydroxyphenylacetic acid. The effect of different doses of the two tropolone compounds on the concentration of homovanillic acid is shown in Fig. 2 and the effect on the concentration of 3,4-dihydroxyphenylacetic acid is shown in Fig. 3.

Figure 2 shows that both drugs reduce the concentration of homovanillic acid, 1 hr after the administration of L-DOPA, and that the reduction bears a linear relation to the logarithm of the dose of the drug used. Figure 3 shows that the concentration of 3,4-dihydroxyphenylacetic acid at first increases with increasing doses of tropolone, but that with the higher doses of both tropolone and tropolone-4-acetamide the concentration of the acid tends to fall. A comparison of the abilities of tropolone and tropolone-4-acetamide to reduce the concentration of homovanillic acid shows that, on a weight basis, tropolone is 6·5 times as active as tropolone-4-acetamide. No homovanillic acid could be detected when tropolone was injected in a dose of 100 mg/kg or more.

*In normal mice treated with probenecid*

Normal mice were injected with tropolone or tropolone-4-acetamide intraperitoneally. Immediately afterwards, they were given probenecid (200 mg/kg intraperitoneally) to prevent the egress of acid metabolites from the brain (Neff, Tozer & Brodie, 1964; Sharman, 1966).

The animals were killed 1·5 hr later and the striata dissected out and analysed for homovanillic acid and 3,4-dihydroxyphenylacetic acid. The striata from mice treated only with probenecid (200 mg/kg intraperitoneally; 1·5 hr previously) were examined at the same time. The experiment was designed as a four-point assay to compare the effects of the two tropolone compounds on the concentrations of the two acid metabolites. The result is illustrated by Fig. 4 which shows that both tropolone and tropolone-4-acetamide reduce the concentration of homovanillic acid in mice treated with probenecid and that tropolone is 3·5 times as active as tropolone-4-acetamide in this test.

**Discussion**

The enzyme, catechol-O-methyl transferase (COMT), is of importance in the metabolism of dopamine in the central nervous system because the major metabolic product of this amine in the brain appears to be homovanillic acid. The inhibition of this enzyme by tropolone derivatives was postulated and confirmed by Belleau & Burba (1961, 1963), who also showed that these substances could block sympathetic $\beta$-receptors, though this observation was not confirmed by Ross & Haljasmaa (1964b).

Tropolone and related compounds have been used in studying the effect of inhibition of COMT on the metabolism of radioactive catecholamines in the brain (Goldstein, 1964), but there have been few comparisons of the ability of different

substances to inhibit the enzyme in the central nervous system of living animals. Carlsson, Corrodi & Waldeck (1963) examined a series of compounds, including tropolone-4-acetamide, by determining the dose of each substance which would reduce the cerebral concentration of 4-hydroxy-3-methoxyphenylethylamine (methoxytyramine), without reducing the cerebral concentration of dopamine, in mice treated with L-DOPA and the monoamine oxidase inhibitor, nialamide. Ross & Haljasmaa (1964b) have measured the COMT activity in supernatants obtained after centrifuging homogenates of brains obtained from mice treated with tropolone derivatives and other compounds. A dose of tropolone-4-acetamide 500 mg/kg was required for these effects. This dose was found by Carlsson, Corrodi & Waldeck (1963) to reduce the concentration of methoxytyramine to below 12% of its normal value and by Ross & Haljasmaa (1964b) to reduce the COMT activity in brain extracts by 50%. The present experiments show that tropolone-4-acetamide prevents the accumulation of homovanillic acid after L-DOPA, presumably as a result of the inhibition of COMT, at dose levels much lower than 500 mg/kg, and that tropolone itself is more active than tropolone-4-acetamide.

In mice treated with L-DOPA, the administration of small doses of tropolone causes a reduction in the concentration of homovanillic acid with a concomitant increase in the concentration of 3,4-dihydroxyphenylacetic acid. The increase in the latter is larger than the decrease in the former and suggests that the rate constant for the removal of 3,4-dihydroxyphenylacetic acid from mouse brain is smaller than that for the removal of homovanillic acid; in other words, it is more difficult for 3,4-dihydroxyphenylacetic acid to get out of the mouse brain than it is for homovanillic acid. After higher doses of tropolone derivatives there is apparently some inhibition of the accumulation of 3,4-dihydroxyphenylacetic acid. The tropolone compounds will inhibit the hydroxylating enzymes involved in the biosynthesis of catecholamines, tropolone-4-acetamide being less active than $\alpha$-propyldihydroxyphenylacetamide (Carlsson *et. al.*, 1963), but there is no evidence to show that they will inhibit DOPA-decarboxylase, the enzyme involved in the formation of dopamine from L-DOPA. It may be that the larger doses of tropolone interfere with the transport of L-DOPA or its metabolites in the brain. The tropolones can also chelate divalent metal ions (Bryant, Fernelius & Douglas, 1953) and thus the higher concentrations may interfere with many metabolic reactions and processes.

It can be concluded that observations on cerebral metabolism of catecholamines made *in vivo* with doses of tropolone compounds in excess of 100 mg/kg should not, in the mouse, be referred simply to the inhibition of COMT.

The present results show that quite small doses of tropolone and tropolone-4-acetamide will reduce the concentration of homovanillic acid in the striatum of normal mice, not treated with L-DOPA, but given probenecid to prevent the active transport of acid metabolites out of the brain. The reduction in the concentration of homovanillic acid again, is directly proportional to the logarithm of the dose of the COMT-inhibitor. In this test, tropolone-4-acetamide was more active relative to tropolone than in the first test, and it can be inferred that the test using probenecid-treated mice gives a better assessment of the inhibition of COMT at the sites where it is normally acting. In this experiment the increase in 3,4-dihydroxyphenylacetic acid was smaller than the fall in homovanillic acid and was similar for all the doses of the tropolones. This is unlikely to be a result of an accelerated removal of 3,4-dihydroxyphenylacetic acid because of the probenecid treatment, which was intended to

block the active transport of acid metabolites from the brain. The finding suggests that in the normal mouse the formation of 3,4-dihydroxyphenylacetic acid is not a simple alternative to the formation of homovanillic acid in the metabolism of dopamine in the striatum, but if the test is to be used as a method for examining the ability of compounds to inhibit catechol-O-methyl transferase, there should be no decrease below the normal concentration of 3,4-dihydroxyphenylacetic acid, because this would indicate an interference in the metabolism of dopamine elsewhere in the metabolic pathway.

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# Turnover of amines using probenecid to block egress of metabolites

D. F. SHARMAN

*Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge, England*

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In 1964 Neff, Tozer & Brodie described a specialized transport system for the removal of 5-hydroxyindol-3-ylacetic acid (5-HIAA), an acid metabolite of 5-hydroxytryptamine (5-HT) from the brain of the rat. The presence of such a system in this species was deduced from the observation that when rats were given probenecid there was an increase in the concentration of 5-HIAA in the brain.

Probenecid is a competitive inhibitor of acid transport in the renal tubules and is thought to act by interfering with the formation of an intermediate complex involved in the transport of acids through the renal tubular cell.

An active transport system for the removal of acid metabolites from the brain is also present in the mouse (Sharman, 1966) and the pigeon (Ahtee, personal communication) but not in the rabbit (Werdinius, 1967, 1968) and is also concerned with the removal of 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid; HVA), an acid metabolite of 3,4-dihydroxyphenylethylamine (dopamine) (Sharman, 1966; Werdinius, 1966) from the brain. The concentration of another acid metabolite of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) is, however, unaffected when probenecid is administered to mice (Sharman, unpublished).

The presence of such an active transport system in the rat and the mouse and the possibility of its inhibition by probenecid has enabled studies to be made on the rates at which acid metabolites of monoamines are formed in the brain. Neff, Tozer & Brodie (1967) have shown that the rate at which 5-HIAA accumulates in the brain, after a maximally effective dose of probenecid, is similar to the rates at which 5-HT and 5-HIAA are synthesized in normal and reserpine-treated rats. It should be emphasized at this point that although the rate of accumulation of an acid metabolite after treatment with probenecid can, in some circumstances, be shown to be similar to the rate of metabolism of the parent monoamine and to the rate of formation of the acid itself, this need not always be so. The possibility of an alternate pathway for the metabolism of the monoamine and an alternative route for the disposition of the acid must not be ignored. Neff, Tozer & Brodie (1967) have obtained evidence that these possibilities do not occur in their experiments on 5-HIAA in the rat brain and that the accumulation of 5-HIAA can be taken as a measure of the rate of turnover of 5-HT. In the rat, the accumulation of 5-HIAA after a maximally effective dose of probenecid is linear for at least 90 min and in the mouse for at least 60 min.

Estimated rates of accumulation, after probenecid, of 5-HIAA in the brain of the rat are 0.40 $\mu$g/g per hr (Neff, Tozer & Brodie, 1967) and 0.32 $\mu$g/g per hr (Diaz, Ngai & Costa, 1968) and in the mouse brain 0.54 $\mu$g/g per hr. It has been

shown in rat brain that reserpine (Neff, Tozer & Brodie, 1967) and ether anaesthesia (Diaz, Ngai & Costa, 1968) increase the rate of accumulation of 5-HIAA after probenecid, which indicates an increased rate of turnover of 5-HT.

The application of similar methods to the study of the rate of metabolism of catecholamines in the brain has proved to be more difficult. The presence in the brain of an acid metabolite of noradrenaline, the concentration of which can be increased by the administration of probenecid has yet to be demonstrated. The effect of probenecid on the acid metabolites of dopamine has, however, proved useful in studying drug-induced changes in dopamine metabolism in the striatum of the mouse (Sharman, 1966 ; Sharman, 1967).

The quantitation of the turnover rate of dopamine from studies on the accumulation of homovanillic acid is not as simple as for 5-HT because there are alternative pathways for the metabolism of dopamine. Also in preliminary experiments on the mouse striatum the molar turnover rate of dopamine, calculated from the fall in the concentration of this amine in the striatal tissues of mice treated with $\alpha$-methyl-$p$-tyrosine, was approximately twice the molar rate of accumulation of homovanillic acid after a maximally effective dose of probenecid. By regarding the active transport system as two enzymic processes in a non-reversible chain (Sharman, 1967) and by partially inhibiting the system with probenecid, it was possible to study whether a drug-induced increase in the concentration of homovanillic acid in the striatal tissues of the mouse was due to an increase in the rate of formation of the acid, to interference with the active transport system or to retention of the acid at a site where the active transport system was not operative.

It seems from such experiments that the homovanillic acid can be present in the striatal tissue in at least two compartments. One of these compartments reflects the homovanillic acid which is in the process of being actively transported out of the brain and another contains homovanillic acid which does not have immediate access to the active transport system. The finding that the concentration of DOPAC is unaffected by probenecid is further evidence in support of this hypothesis (Sharman, unpublished) showing that acid metabolites can be formed at a site where the active transport system does not act. Because of the possibility that homovanillic acid is present at two separate sites in the brain tissue, the application of simple enzyme kinetics to the results obtained after partial inhibition of the active transport by probenecid must be reconsidered, because it was assumed that all the homovanillic acid in the tissue was immediately involved in the active transport system. If, however, changes in the rate of formation of homovanillic acid are reflected in an increase in the concentration of homovanillic acid which is distributed in a constant proportion between the compartments, then the simple approach will still hold. In all the drug-induced increases in the rate of formation of homovanillic acid so far examined, the relative increase in homovanillic acid induced by a dose of probenecid has only rarely exceeded the relative increase induced by the same dose of probenecid in normal mice, whereas the normal relative increase would be expected to be exceeded in all cases if only the homovanillic acid in the process of being actively transported was affected when the rate of synthesis of homovanillic acid is increased. There have been several instances where the probenecid-induced relative increase in homovanillic acid in the striatum of drug-treated mice has been less than in normal mice, indicating that the drug treatment had caused a retention of homovanillic acid in the tissue or that an equilibrium situation had not been attained.

With the apparently simpler situation which prevails for the metabolism of 5-HT, the presence of two or more compartments for 5-HIAA should also be examined. Diaz, Ngai & Costa (1968) have shown that the rate of synthesis of 5-HIAA is increased threefold in rats anaesthetized with ether but that the concentration of 5-HIAA in the brain was increased only 1.7 times. This implies that at a higher concentration of 5-HIAA the active transport system is more efficient, but it is also possible that only a part of the normal concentration of 5-HIAA is involved in the active transport process.

The administration of 2-amino-tetralin to mice results in a reduction in the concentration of 5-HIAA in the brain (Robinson & Sharman, 1966). The results of an experiment in which the effect of probenecid alone and in combination with this drug will serve to illustrate the method of studying the rate of formation of 5-HIAA. Figure 1 shows that after treatment with 2-amino-tetralin the rate of accumulation of 5-HIAA induced in the mouse brain by a maximally effective dose of probenecid is approximately half that seen with normal mice. This result

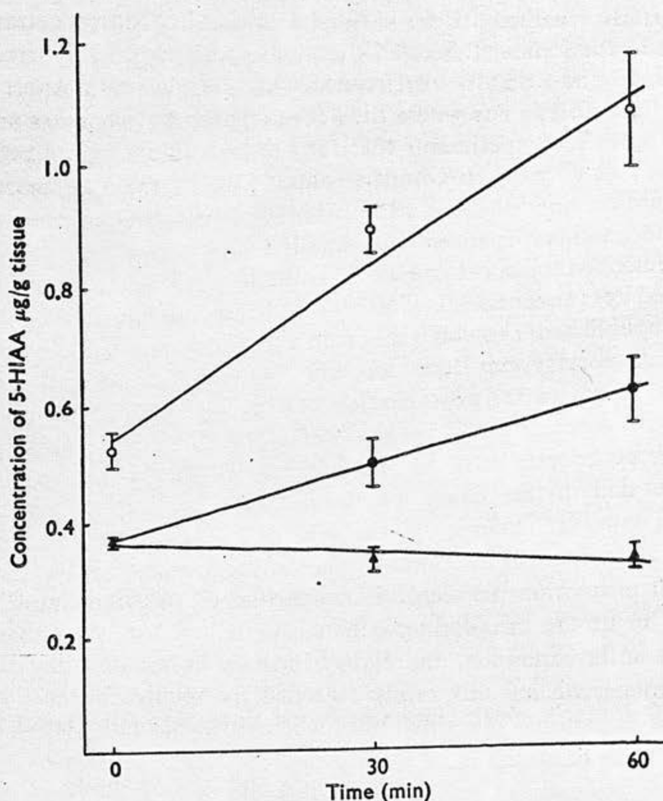


FIG. 1. Effect of 2-aminotetralin on the accumulation of 5-hydroxyindol-3-ylacetic acid (5-HIAA) in the brain of the mouse after treatment with probenecid. ○——○, Concentration of 5-HIAA in the brains of mice given probenecid (200 mg/kg i.p.) at time 0; ▲——▲, concentration of 5-HIAA in the brains of mice given 2-aminotetralin (30 mg/kg i.p.) 60 min before time 0; ●——●, concentration of 5-HIAA in the brains of mice given 2-aminotetralin (30 mg/kg i.p.) 60 min before time 0 and probenecid (200 mg/kg i.p.) at time 0. Concentrations are drawn ± S.E.M.; each point is the mean of at least seven determinations.

suggests that the fall in 5-HIAA concentration after 2-amino-tetralin is a reflection of a reduced rate of formation of the acid.

It can be concluded that the use of probenecid to inhibit the active transport of acid metabolites of monoamines out of the rodent brain will prove to be a most useful aid in the study of the metabolism of monoamines in the brain, but it must be remembered that the effect of probenecid can only be used as an index of the rate of synthesis or arrival of an acid metabolite at a site where the active transport system can function.

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# NORADRENALINE CONTENT IN THE HEART AND SPLEEN OF THE MOUSE UNDER NORMAL CONDITIONS AND AFTER ADMINISTRATION OF SOME DRUGS

BY

D. F. SHARMAN, S. VANOV and MARTHE VOGT

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# NORADRENALINE CONTENT IN THE HEART AND SPLEEN OF THE MOUSE UNDER NORMAL CONDITIONS AND AFTER ADMINISTRATION OF SOME DRUGS

BY

D. F. SHARMAN, S. VANOV AND MARTHE VOGT

*From the A.R.C. Institute of Animal Physiology, Babraham, Cambridge*

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The noradrenaline content of the heart and spleen was investigated in normal mice and in mice treated with drugs. A modification of the methods of Bertler, Carlsson & Rosengren (1958) was used for extraction, and of v. Euler & Floding (1955) for fluorimetric estimation of the amine. In normal mice the mean noradrenaline content of the heart was 0.55 μg/g and that of the spleen 0.26 μg/g fresh tissue. Iproniazid (100 mg/kg), nicotine (0.1 mg/kg) and histamine (0.5 mg/kg), given 1 and 3 hr before killing the mice, did not significantly change the concentration of noradrenaline in the heart. Neither did nicotine and histamine, administered 1 hr before death, significantly alter the noradrenaline content of the spleen. The rapid changes in the catechol amine content of mouse tissues reported with these drugs by De Schaepdryver & Preziosi (1959) were not observed. In contrast, reserpine (2.5 mg/kg), methyl reserpate methyl ether (1 mg/kg), and methyl 18-epireserpate methyl ether (2 mg/kg) caused severe depletion of noradrenaline from the heart and spleen of the mice.

Of the numerous reports on the normal catechol amine content of mammalian tissues few concern mice. The effect of drugs on the amine stores in mice has also been studied very little. In 1959 De Schaepdryver & Preziosi, using adsorption of adrenaline and noradrenaline on aluminium oxide, elution by sulphuric acid and fluorimetric estimation, analysed the catechol amine concentrations of the suprarenal, heart, liver and spleen in normal mice. They found that a number of drugs rapidly deplete the amines from these tissues. Thus, 1 hr after the intraperitoneal injection of nicotine (0.1 mg/kg) or iproniazid (100 mg/kg) complete loss of both amines from the heart was observed. Similarly, 1 hr after administration of nicotine or histamine (0.5 mg/kg), no noradrenaline was detected in the spleen. Very rapid recovery of both cardiac and splenic noradrenaline to normal and to levels above normal was observed within a few hours. Even after reserpine, recovery of the cardiac noradrenaline was nearly complete in 48 hr. However, experiments of several authors (Carlsson, Rosengren, Bertler & Nilsson, 1957 ; Muscholl & Vogt, 1958 ; Paasonen & Krayer, 1958) have shown in other species that, after depletion by reserpine, it took many days for the concentration of tissue noradrenaline to recover. These results suggested that, in contrast to other species, the stores of noradrenaline in mouse tissues are labile and easily affected by drugs. Other species differences in the metabolism and storage of catechol amines have been recently demonstrated by Sanan & Vogt (1962).

The present study was made in order to investigate further what appeared to be unusual behaviour of the tissue catechol amines in the mouse and to repeat the experiments of De Schaepdryver & Preziosi (1958) by using another method of estimation. The noradrenaline content of the heart and spleen was estimated in normal mice and in mice treated with iproniazid, nicotine, histamine, reserpine and two of its analogues.

## METHODS

*Animals and drugs.* Albino mice of both sexes (predominantly male) were used. The body weight of the animals ranged from 20 to 40 g. The animals were kept in groups of 10 or 20 at room temperature. Food in pellets and tap-water were allowed *ad libitum.*

Special precautions were taken to minimize the biological variations related to the origin of the animals. The majority of the experiments were carried out on one breed of mice reared in the laboratories ; for the remaining experiments animals from a single strain were obtained from a dealer. The organs of a group of five or six mice were pooled, since this number provided sufficient tissue for an accurate estimation of noradrenaline. The groups were made as uniform as possible in respect of body weight and sex.

The following drugs were used: iproniazid phosphate (Marsilid, Roche Products), nicotine hydrogen tartrate (British Drug Houses), histamine acid phosphate (British Drug Houses), reserpine (Serpasil, Ciba), methyl reserpate methyl ether hydrochloride and its 18-epi isomeride (Su-8842 and Su-9064, Ciba). The doses of nicotine and histamine are expressed in terms of the base and those of all other drugs as weight of the salts.

All drugs were dissolved in an isotonic solution of sodium chloride and injected intraperitoneally in a volume of 0.5 ml./20 g body weight. Controls were injected with the same volume of sodium chloride solution.

The animals were killed by decapitation and exsanguinated. The hearts were dissected as rapidly as possible. The blood and the blood clots were removed by blotting on filter paper. Before excising the spleens, electrical stimulation of the splenic nerves (square waves of 100 msec duration, 12/sec, 6 V) was carried out for a few sec in an attempt to induce contraction of the organ.

*General procedure.* Immediately after their removal the tissues were weighed and homogenized in 0.1 N hydrochloric acid (2 ml./g tissue) in the presence of a few mg ascorbic acid. The homogenate was diluted with about twice its volume of water. The proteins were precipitated by adding 1 vol. 0.8 N perchloric acid. The further treatment of the extract was essentially the same as described by Bertler *et al.* (1958). The main characteristic of this method is the absorption of the catechol amines on the cation exchange resin Dowex-50$\times$8 in a column. The elution was done by gravity, and 10 ml. of 1.2% hydrochloric acid was used. The eluates were analysed fluorimetrically for noradrenaline with an Aminco-Bowman spectrophotofluorimeter. The trihydroxyindole method of von Euler & Floding (1955) was used in a somewhat modified way: the reaction (oxidation with potassium ferricyanide and rearrangement with alkali) was performed in the dark and the *p*H of the M acetate buffer was 6.5 (Sharman, 1960). Von Euler & Lishajko (1961) improved the original method by addition of ethylene diamine to the mixture of sodium hydroxide and ascorbic acid. In the majority of the estimations, ethylene diamine was used but was incorporated in the sodium hydroxide solution, since this was added separately from ascorbic acid, as suggested by Crawford & Law (1958).

The samples were activated at 400 m$\mu$, and the resulting fluorescence was read at 500 m$\mu$. The catechol amines in the extracts were read against a standard noradrenaline solution treated in the same way as the sample. The results were expressed as $\mu$g of noradrenaline base. Extract blanks were obtained after allowing a portion of the eluates to fade for 7 min in the presence of potassium ferricyanide and sodium hydroxide and adding ascorbic acid afterwards.

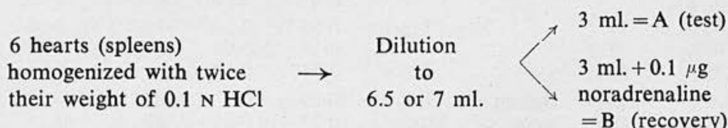
A polished Araldite rod was used as a standard for the calibration of the spectrophotofluorimeter. The sensitivity was checked and corrected if necessary before each reading.

All solutions for the fluorimetric analysis were made with deionized water. Also, all glassware was rinsed with deionized water in order to prevent possible interference by some fluorescent material contaminating the ordinary distilled water.

*Test for sensitization or masking.* To 1 ml. of each eluate a known amount of noradrenaline was added. The fluorescence was developed in the usual way, and the increase in fluorescence above that obtained with 1 ml. of eluate only was measured. This increase should theoretically be identical with the fluorescence produced by the added amount of noradrenaline in pure solution. Any deviation from the expected reading would disclose either masking or intensification of the reaction by some material in the tissue. Masking was hardly ever found, but the fluorescence produced in the extracts was sometimes slightly higher than in pure solution; the difference never exceeded the equivalent of 2.0 ng noradrenaline.

*Recovery.* The recovery was checked in every single experiment. For this purpose two portions of 3 ml. each were pipetted off from the homogenates which had been diluted with water to either 6.5 or 7 ml. To one of these portions 0.1 μg of noradrenaline was added. The recovery was determined by comparing the fluorescence of the two portions. The mean recoveries were of the order of 80%.

*Sensitivity.* Because of these various controls, the fluorescence was measured on about 3/70th of the original homogenate, as seen from the following plan:




A and B each yielded 10 ml. eluates, which were used as follows:

2 ml. for duplicate estimation on 1 ml. each;

2 ml. for duplicate check on masking or sensitization (1 ml. eluate + known amount of noradrenaline);

1 ml. for faded (extract) blank.

If 1 g of tissue is worked up, reliable readings can be obtained by this procedure down to a tissue concentration of noradrenaline of 0.1 μg/g. Estimations of lower concentrations are only approximate.

## RESULTS

*The noradrenaline content of the heart and spleen in normal mice.* The animals referred to here as "normal" are either untreated mice or controls injected with 0.9% sodium chloride solution. The noradrenaline content of the heart and spleen for these is summarized in Table 1. The penultimate column shows the individual concentrations, means, standard deviations and standard errors of the mean, corrected for recovery. The percentage recovery will be found in the preceding column.

The coefficient of variation was not significantly changed when the concentrations were expressed as μg noradrenaline/100 g body weight instead of μg/g fresh tissue. No consistent relationship was found between body weight and noradrenaline concentration. Since estimations of noradrenaline concentrations below 0.1 μg/g tissue have a low degree of accuracy, the second decimal in the table is only an approximation.

TABLE 1

NORADRENALINE CONTENT OF THE HEART AND SPLEEN IN NORMAL MICE

s.d.=standard deviation. s.e.=standard error of the mean

| Group | No. of animals and sex | Total body wt. in g | Tissue | Total wt. of tissue in mg | Noradrenaline μg/g tiss. | Noradrenaline μg/100 g body wt. | Recovery % | Noradrenaline, μg/g tissue, corrected for recovery | Remarks |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 6 ♂ | 183 | Heart | 860 | 0·45 | 0·21 | 63 | 0·71 | |
| 2 | 6 ♂ | 166 | Heart | 850 | 0·37 | 0·18 | 65 | 0·57 | No |
| 3 | 6 ♂ | 231 | Heart | 1046 | 0·52 | 0·23 | 92 | 0·56 | treatment |
| 4 | 6 ♂ | 237 | Heart | 1072 | 0·53 | 0·24 | 87 | 0·61 | |
| | Mean | 204 | | 957 | 0·47 | 0·21 | 76·7 | 0·61 | |
| | s.d. | | | | 0·07 | 0·026 | | 0·07 | |
| | s.e. | | | | 0·035 | 0·013 | | 0·03 | |
| 5 | 6 ♂ | 229 | Heart | 1006 | 0·56 | 0·24 | 96 | 0·58 | |
| 6 | 5 ♂ | 223 | Heart | 936 | 0·59 | 0·25 | 71 | 0·83 | |
| 7 | 6 ♂ | 214 | Heart | 904 | 0·39 | 0·16 | 60 | 0·65 | |
| 8 | 6 ♂ | 233 | Heart | 1163 | 0·59 | 0·29 | 70 | 0·84 | Saline- |
| 9 | 6 ♀ | 194 | Heart | 914 | 0·59 | 0·27 | 108 | 0·55 | treated |
| 10 | 6 ♀ | 185 | Heart | 845 | 0·84 | 0·37 | 95 | 0·88 | controls |
| 11 | 6 ♀ | 186 | Heart | 890 | 0·72 | 0·34 | 88 | 0·82 | |
| 12 | 6 ♂ | 176 | Heart | 895 | 0·43 | 0·21 | 69 | 0·61 | |
| | Mean | 205 | | 944 | 0·59 | 0·28 | 82·1 | 0·72 | |
| | s.d. | | | | 0·14 | 0·07 | | 0·13 | |
| | s.e. | | | | 0·05 | 0·03 | | 0·05 | |
| | | | | Overall mean | 0·55 | 0·26 | 80·3 | 0·68 | |
| | | | | s.d. | 0·14 | 0·06 | | 0·13 | |
| | | | | s.e. | 0·04 | 0·02 | | 0·04 | |
| 1 | 6 ♀ | 194 | Spleen | 943 | 0·29 | 0·13 | 100 | 0·29 | |
| 2 | 6 ♀ | 186 | Spleen | 1263 | 0·21 | 0·13 | 82 | 0·25 | Saline- |
| 3 | 6 ♀ | 188 | Spleen | 860 | 0·33 | 0·15 | 103 | 0·32 | treated |
| 4 | 5 ♀ | 147 | Spleen | 690 | 0·38 | 0·17 | 69 | 0·55 | controls |
| 5 | 6 ♂ | 195 | Spleen | 979 | 0·26 | 0·12 | 65 | 0·40 | |
| 6 | 6 ♂ | 171 | Spleen | 1006 | 0·17 | 0·09 | 75 | 0·22 | |
| 7 | 6 ♂ | 147 | Spleen | 922 | 0·16 | 0·09 | 53 | 0·30 | |
| | Mean | 176 | | 952 | 0·26 | 0·12 | 78·1 | 0·33 | |
| | s.d. | | | | 0·08 | 0·03 | | 0·11 | |
| | s.e. | | | | 0·03 | 0·01 | | 0·04 | |

The mean for the noradrenaline content of the *heart* of the untreated mice was 0.47 μg/g tissue with a standard deviation of $\pm 0.07$ (Table 1). The corresponding figures for the saline-treated controls were $0.59 \pm 0.14$ μg/g, and the mean of both groups $0.55 \pm 0.14$ μg/g. The difference between the two groups becomes smaller (and non-significant) when correction is made for recovery (Table 1, penultimate column).

The *spleen* was only analysed in saline-treated mice. The mean noradrenaline concentration in seven groups was $0.26 \pm 0.08$ μg/g tissue. This figure is lower than that obtained by De Schaepdryver & Preziosi (0.686 μg/g tissue). In order to obtain the highest concentration possible, the splenic nerves were stimulated for a few sec so as to reduce the spleen volume ; not much contraction was, however, observed, probably because the spleen was already contracted as a result of the exsanguination.

The results both for the heart and spleen show considerable variations which are reduced by correcting for recovery. Consistent differences between the two sexes were not found, but an occasional group of female mice had a slightly higher noradrenaline content of their spleens.

*The noradrenaline content of the heart and spleen of the mouse after administration of drugs.* The effects of the drugs studied are presented in Table 2. These drugs might be conveniently divided into two groups. Firstly, iproniazid, nicotine and histamine, and, secondly, reserpine and two of its analogues—methyl reserpate methyl ether and methyl 18-epireserpate methyl ether. The effect of reserpine served to confirm that the method would demonstrate a depletion of catechol amines if this were to occur.

TABLE 2

NORADRENALINE CONTENT OF THE HEART AND SPLEEN OF THE MOUSE AFTER TREATMENT WITH DRUGS

| Group | No. of animals and sex | Total body wt. in g | Tissue | Total wt. of tissue in mg | Dose of drug (mg/kg) | Duration of exp. (hr) | Noradrenaline µg/g tiss. | Noradrenaline µg/100 g body wt. | Recovery % |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 6 ♂ | 214 | Heart | 1002 | Iproniazid, 100 | 1 | 0·54 | 0·25 | 87 |
| 2 | 6 ♂ | 252 | Heart | 1002 | | 1 | 0·64 | 0·25 | 80 |
| 3 | 6 ♂ | 217 | Heart | 894 | | 1 | 0·31 | 0·12 | 100 |
| 4 | 6 ♂ | 227 | Heart | 981 | | 3 | 0·60 | 0·26 | 85 |
| 5 | 6 ♀ | 190 | Heart | 863 | Nicotine, 0·1 | 1 | 0·74 | 0·33 | 134 |
| 6 | 6 ♀ | 185 | Heart | 891 | | 1 | 0·66 | 0·31 | 77 |
| 7 | 5 ♀ | 141 | Heart | 659 | | 1 | 0·76 | 0·35 | 92 |
| 8 | 6 ♂ | 180 | Heart | 832 | | 3 | 0·50 | 0·22 | 70 |
| 9 | 6 ♂ | 165 | Heart | 978 | Histamine, 0·5 | 1 | 0·35 | 0·20 | 62 |
| 10 | 6 ♂ | 154 | Heart | 765 | | 1 | 0·48 | 0·23 | 78 |
| 11 | 6 ♂ | 239 | Heart | 917 | Reserpine, 2·5 | 18 | 0·013 | 0·005 | 58 |
| 12 | 6 ♂ | 235 | Heart | 1058 | Su-8842, 1 | 2 | 0·03 | 0·01 | 93 |
| 13 | 6 ♂ | 194 | Heart | 1052 | Su-8842, 1 | 2 | 0·03 | 0·01 | 80 |
| 14 | 6 ♂ | 242 | Heart | 1264 | Su-9064, 2 | 2 | 0·03 | 0·01 | 136 |
| 1 | 5 ♀ | 145 | Spleen | 708 | Nicotine, 0·1 | 1 | 0·34 | 0·16 | 105 |
| 2 | 6 ♂ | 180 | Spleen | 797 | Nicotine, 0·1 | 3 | 0·30 | 0·12 | 68 |
| 3 | 6 ♂ | 165 | Spleen | 1160 | Histamine, 0·5 | 1 | 0·15 | 0·10 | 63 |
| 4 | 6 ♂ | 194 | Spleen | 960 | Su-8842, 1 | 2 | Less than 0·01 | | 61 |

Table 2 shows that iproniazid, nicotine and histamine in the doses used did not produce significant changes in the noradrenaline content of the heart and spleen in the course of 1 or 3 hr.

In contrast with this, a single injection of reserpine provoked in 18 hr a profound fall in the noradrenaline content of the heart. Similarly, the two reserpine analogues, methyl reserpate methyl ether and its 18-epi isomeride, caused severe loss of noradrenaline from heart and spleen. In confirmation of Robison, Lucas, MacPhillamy, Barrett & Plummer (1961), the action of these compounds was found to be very fast, negligible amounts of the amine being left in the tissues after as little as 2 hr. The behavioural and autonomic effects of the doses used were less marked than those of reserpine, 2.5 mg/kg. There was obvious sedation and reduced reactivity to external stimuli, closure of the eyelids and tremor.

## DISCUSSION

As Udenfriend (1959) stated, specificity and sensitivity are the most important criteria in the choice of a chemical method for the estimation of catechol amines. The specificity of the present method is based on the combination of selective adsorption and elution with a characteristic fluorescence. Of the catechol amines which might be mistaken for noradrenaline, isoprenaline has never been found in

the tissues examined, while adrenaline is present in such small amounts that it cannot seriously affect the results. The fluorescence derived from dopamine with this method is less than 1% of that derived from noradrenaline (Crawford & Law, 1958).

The sensitivity of the method is sufficient to measure accurately 0.1 μg noradrenaline in 1 g of fresh tissue, and is, therefore, adequate for the estimation of noradrenaline in 1 g of normal heart which is about 5 times that figure. Since the concentration of adrenaline in all mammalian species examined, including the rat, is only a few per cent. of that of noradrenaline, it was obvious that reliable figures for the adrenaline content of the organs of mice could not be expected with this method. In addition, it is known that the concentration of adrenaline in tissues supplied with sympathetic nerves varies much more than that of noradrenaline. No attempt was therefore made to estimate any adrenaline in the tissues analysed.

As a precaution, recoveries were carried out in each estimation to check on accidental losses. Though, on the average, the recovery was of the order of 80%, the variation of the losses suggests that the precision is greatly increased by estimating the recovery from each homogenate.

The noradrenaline content of the heart of normal mice found in the present work (overall mean 0.55 μg/g) is in good agreement with the figures reported by De Schaepdryver & Preziosi (1959) and by Porter *et al.* (1961).

The concentration in the spleen of normal mice, however (0.26 μg/g), was significantly lower than that found by De Schaepdryver & Preziosi (0.69 μg/g). In order to stabilize the weight of the spleen by expelling as much blood as possible, electrical stimulus was applied for a few sec to the splenic pedicle in the present experiments. It is most unlikely that this should have reduced the noradrenaline content of the splenic tissue, since Vogt (1954) has shown that stimulation of the superior cervical sympathetic nerve for 2 hr does not change the noradrenaline content of the ganglion. The reason for the lower figure may lie in a strain difference.

After reserpine and two of its analogues, the noradrenaline content of the heart and spleen fell to values so low that they were not significantly different from zero. In this the present results agree with those of De Schaepdryver & Preziosi with reserpine. When, however, iproniazid, nicotine and histamine were injected, and the tissues examined 1 and 3 hr later, the results obtained were at variance with those of De Schaepdryver & Preziosi (1959). These authors found no noradrenaline in the heart after iproniazid and nicotine, and none in the spleen after nicotine and histamine, whereas the foregoing experiments gave normal concentrations for the noradrenaline in all these instances. It is very difficult to explain these discrepancies. Our conclusion is that the noradrenaline content of the mouse organs is not changed more readily than that of other species and that the stores are just as stable in the mouse as in the rabbit or cat.

Nicotine and histamine are drugs which deplete catechol amines from the adrenal gland by a direct action on the medullary tissue. They are also known to cause stimulation of sympathetic ganglia. The present experiments with these drugs show that such stimulation is not accompanied by a depletion of the amines from their

peripheral storage sites. This is in agreement with the findings of Muscholl (1961) on the effect of nicotine and dimethylphenylpiperazinium iodide on cat atria, and of Sanan & Vogt (1962) with the latter drug on sympathetic ganglia.

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# IONTOPHORETIC RELEASE OF ADRENALINE, NORADRENALINE AND 5-HYDROXYTRYPTAMINE FROM MICROPIPETTES

BY

K. KRNJEVIĆ, R. LAVERTY and D. F. SHARMAN

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# IONTOPHORETIC RELEASE OF ADRENALINE, NORADRENALINE AND 5-HYDROXYTRYPTAMINE FROM MICROPIPETTES

BY

K. KRNJEVIĆ, R. LAVERTY* AND D. F. SHARMAN

*From the Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge*

(*Received March* 10, 1963)

Estimations have been made of the amounts of adrenaline, noradrenaline and 5-hydroxytryptamine released by iontophoresis from micropipettes. In most experiments, the amount released was linearly related to the total electrical charge, but the transport numbers for different pipettes, filled with samples of the same solutions of adrenaline or of noradrenaline, varied substantially. Two pipettes containing noradrenaline failed to release any appreciable amounts of the drug. The transport number of 5-hydroxytryptamine was relatively constant (mean, 0.14).

The iontophoretic method of applying sympathomimetic drugs to neurones in the central nervous system is being used more and more extensively (Curtis & Davis, 1962 ; Bradley & Wolstencroft, 1962 ; Krnjević & Phillis, 1963a, b). It is therefore of interest to know how much drug is released from a micropipette by iontophoresis. Studies, made with pipettes containing acetylcholine chloride, have shown that the average fraction of the total current carried by ionized acetylcholine (its transport number) in such pipettes is not very different from that observed with a large volume of solution, but there was a substantial variation in behaviour between one pipette and another (Krnjević, Mitchell & Szerb, 1963).

It is preferable to study the iontophoretic release of drugs by currents which are at least similar in magnitude to those used in experiments on nerve cells. Inevitably only very small amounts of drugs are thus available for estimation. We have now examined the iontophoresis of adrenaline, noradrenaline and 5-hydroxytryptamine, for which adequately sensitive methods of assay were available.

## METHODS

The micropipettes consisted of five Pyrex glass tubes sealed together and pulled out to a fine tip, as described by Krnjević & Phillis (1963a). They were similar to those used in previous studies of cerebral cortical neurones (Krnjević & Phillis, 1963a, b). The compound tips had outside diameters of 6 to 12 $\mu$.

In the present experiments, only one of the barrels was filled with a concentrated solution of a drug, the others containing merely water. The solutions were prepared 48 hr before the experiments to allow time for diffusion to the tips. The concentrations of drugs were as

* Beit Memorial Fellow.

follows: 0.13 M-5-hydroxytryptamine creatinine sulphate (May & Baker); 0.75 M-adrenaline acid tartrate (British Drug Houses); and 1.7 M-noradrenaline (British Drug Houses) acidified with concentrated hydrochloric acid. All three solutions had a *p*H between 3 and 4.

The pipettes were held vertically, with the tip dipping in 1.0 ml. of 150 mM-NaCl, to which was added either a trace of ascorbic acid (about 10 mg/100 ml.) to preserve 5-hydroxytryptamine, or hydrochloric acid (giving a final concentration of 2 mM) to preserve the catechol amines.

The iontophoretic currents were measured by a series galvanometer, with an accuracy of 1 nA ($10^{-9}$ A), and there was a 50 M$\Omega$ resistor in series with the micropipette to reduce fluctuations in the current. The latter was kept at 100 nA in all experiments, well within the range of currents used when testing nerve cells. Four to six different periods of release (lasting up to 10 min) were tried with each micropipette, so that the relation between the release and the total electrical charge could be examined over a substantial range of times. Samples of fluid were also collected during periods when the drugs were allowed to diffuse out spontaneously, to estimate the magnitude of any spontaneous leak.

All samples were assayed within 2 to 3 hr and, for controls, several solutions of known concentration were handled in the same way to test for any possible loss of drug or contamination during handling; none could be detected.

*Estimations.* Noradrenaline and adrenaline were estimated fluorimetrically as described by Sharman, Vanov & Vogt (1962), but a Locarte filter fluorimeter was used. For noradrenaline, the primary filter was an LF/2 and the secondary an Ilford No. 625; for adrenaline the primary filter was a Corning 3880 with a half standard thickness Corning 3113, and the secondary a Chance OY4. The accuracies of this method are about $\pm 10\%$ in the upper range of concentrations for noradrenaline and about $\pm 5\%$ for adrenaline. The lower limits of accurate estimation are about 50 and 20 pmole for noradrenaline and adrenaline respectively. Hence, the lowest values in our results are only very approximate, being liable to errors approaching $\pm 100\%$.

5-Hydroxytryptamine was estimated by its fluorescence in 3 N-HCl (Udenfriend, Bogdanski & Weissbach, 1955) using an Aminco-Bowman spectrofluorimeter. The lower limit of estimation was about 10 pmole.

## RESULTS

In all, twelve multibarrelled micropipettes were used, four containing adrenaline, four noradrenaline and four 5-hydroxytryptamine solution.

The amount of iontophoretic release of drug was plotted against the electrical charge after making due correction for any spontaneous leakage of drug. The general relation between the release of drug and the charge was obtained from the slopes of lines drawn through the experimental points. Examples of such graphs are shown in Figs. 1 and 2. The effective transport number was calculated by multiplying the slope (expressed in mole/coulomb) by 96,500.

### *Adrenaline*

The values of the transport numbers were 0.13, 0.14, 0.20 and 0.37 (mean, 0.21). The full range of slopes is illustrated by the lines in Fig. 1. It can be seen that there was relatively little scatter of points obtained with any one pipette, and that all the lines, when extrapolated, went through or near the origin.

The marked difference between the slopes was associated with corresponding variations in tip diameter, electrical resistance and, especially, in the amount of

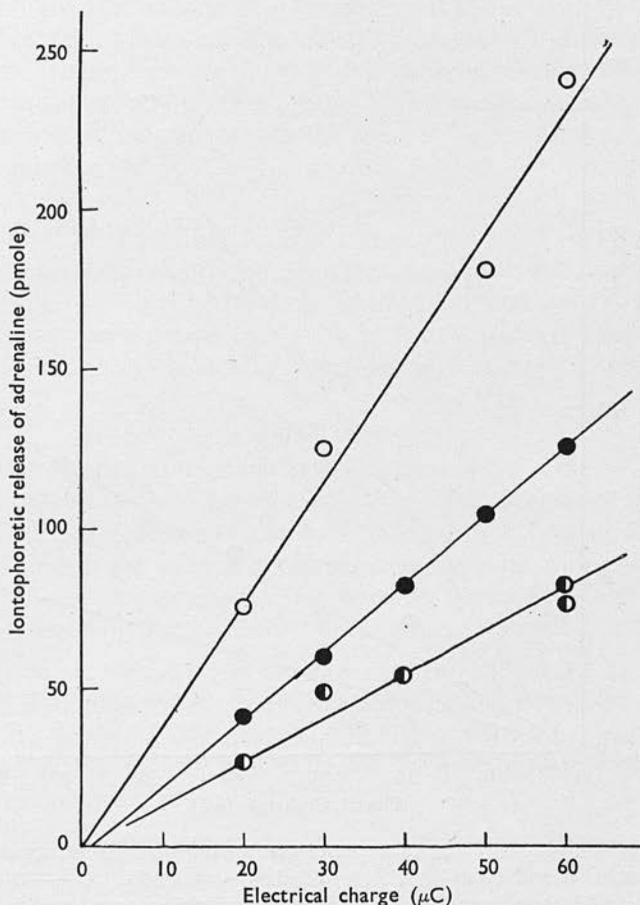


Fig. 1. The relationship between iontophoretic release of adrenaline (ordinate, pmole) and electrical charge (abscissa, μcoulomb) for three different micropipettes containing adrenaline acid tartrate. The current was 100 nA. Straight lines were positioned by eye.

spontaneous leak of adrenaline. Thus the smallest rate of release was from a pipette with a 7 μ tip (overall outside diameter) and a resistance much greater than 100 MΩ, and with a spontaneous leak of only 0.3 pmole/min; whereas the steepest slope was given by a 12 μ diameter pipette, with a tip resistance of 70 MΩ and a leak of 25 pmole/min.

*Noradrenaline*

Two micropipettes allowed current to flow without any difficulty; the values of the transport numbers were 0.34 and 0.37 and the spontaneous leakage was 0.5 to 1 pmole/min. The experimental points obtained with one of these pipettes are shown in Fig. 2 (o—o).

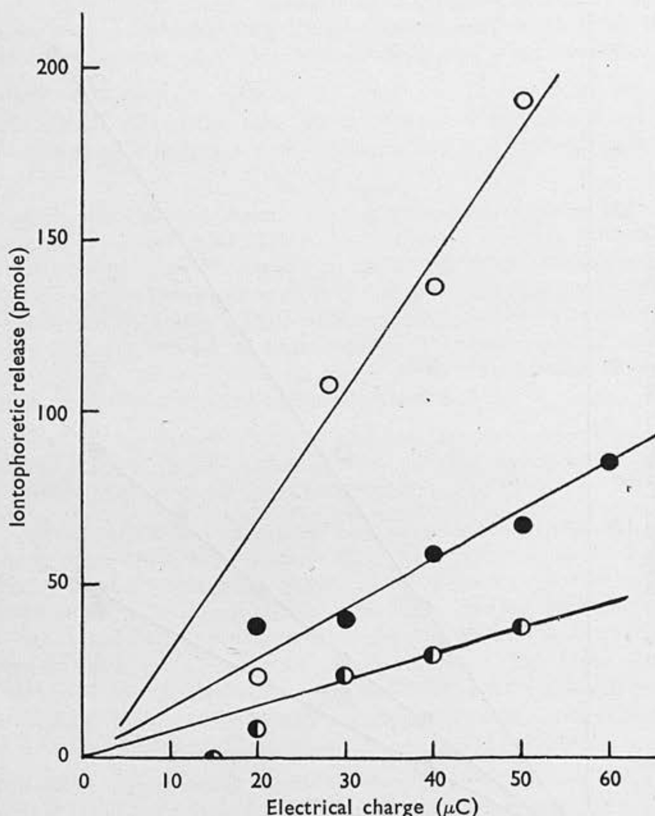


Fig. 2. The relationship between iontophoretic release (ordinate, pmole) of noradrenaline and of 5-hydroxytryptamine and electrical charge (abscissa, $\mu$coulomb). ○——○ and ◐——◐: release of noradrenaline from two micropipettes containing noradrenaline hydrochloride. ●——●: release of 5-hydroxytryptamine from another pipette containing 5-hydroxytryptamine creatinine sulphate. The iontophoretic current was 100 nA in all instances. Straight lines were positioned by eye.

The other two pipettes did not behave so satisfactorily: current did not flow regularly because of a large and variable increase in the tip resistance. Hence much larger voltages had to be applied to keep the current flow at 100 nA (about 100 V instead of the usual 10 V). Moreover, no spontaneous leak of drug could be detected, so any leak was probably less than 0.1 pmole/min. These two pipettes did not release noradrenaline in a way clearly related to the iontophoretic current. One of them was tested on two different occasions: the first time there was only a minimal release of noradrenaline, which apparently did not vary with the amount of charge; on the second occasion the release apparently increased somewhat with the charge (points ◐—◐ in Fig. 2). However, as all these estimates were at the lower limit of sensitivity of the method, it is not certain that there was any significant release of noradrenaline. The transport number was probably in the range of 0.0 to 0.07. The other pipette giving only a doubtful release of noradrenaline gave a transport

number in the range 0.0 to 0.02 ; but, after several applications of a high voltage (with reversal of the polarity from time to time), the same pipette appeared to conduct a current more readily, and the value of the transport number rose to 0.17 (this same procedure applied to the other pipette did not change its behaviour). In this instance there was no obvious correlation between tip diameter or resistance and the transport number.

*5-Hydroxytryptamine*

The four pipettes containing the 5-hydroxytryptamine solution behaved in the most consistent manner. A typical result is indicated in Fig. 2 (●—●). The four values of the transport numbers were 0.10, 0.13, 0.14 and 0.18 (mean, 0.14). The spontaneous leak of drug was usually 1 to 2 pmole/min.

## DISCUSSION

The most significant result of the present experiments is the demonstration of substantial variations in the effective transport number determined when a similar current flows through samples of the same solution of a drug in different micropipettes, even though the latter were prepared at the same time and from similar glass tubing. This result was shown, for instance, by the variations in the release of adrenaline from the pipettes containing solutions of adrenaline acid tartrate.

The mean value of the transport numbers for adrenaline was 0.21, which is only about two-thirds of that for the release of acetylcholine from solutions of acetylcholine chloride (Krnjević *et al.*, 1963). The lower mean value and the scatter of results with adrenaline may be due to variations in ionization of the acid tartrate in the tip of the pipette.

The micropipettes containing 5-hydroxytryptamine showed least variation in transport numbers, which were consistently relatively low. This result supports the suggestion that the creatinine component in solutions of 5-hydroxytryptamine creatinine sulphate probably carries one-half of the iontophoretic current (Curtis & Davis, 1962). However, it does not seem safe to assume on this basis that 5-hydroxytryptamine has double the potency of other drugs, as these (such as adrenaline) may have a similar transport number.

The experiments with noradrenaline presented a special problem, since the micropipettes either released a relatively large amount of drug or practically none. Use of such pipettes giving extremely low values of transport number could be very misleading when testing nerve cells since, although the micropipettes would be conducting the appropriate current, hardly any noradrenaline would leave their tips. It must be supposed that under these conditions the tip behaves as a semipermeable membrane (perhaps because it is blocked by some contaminating particle) which allows the passage of hydrogen or chloride ions but not those of noradrenaline.

Evidently one cannot assume that iontophoresis is a strictly quantitative method of application of drugs when comparing different compounds, and it is clearly essential to make observations with several pipettes before drawing any general conclusions about the effectiveness of any drug.

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# ACTION OF 2, 4, 5-TRIHYDROXYPHENYLETHYLAMINE ON THE STORAGE AND RELEASE OF NORADRENALINE

BY

R. LAVERTY, D. F. SHARMAN and MARTHE VOGT

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# ACTION OF 2, 4, 5-TRIHYDROXYPHENYLETHYLAMINE ON THE STORAGE AND RELEASE OF NORADRENALINE

BY

R. LAVERTY,* D. F. SHARMAN AND MARTHE VOGT

*From the Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge*

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Porter, Totaro & Stone (1963) have reported that a single injection of 6-hydroxydopamine (2,4,5-trihydroxyphenylethylamine) lowered the noradrenaline content of the mouse heart by more than 50% for longer than 25 days. Porter *et al.* (1963) proposed that this prolonged action was due to destruction of the noradrenaline binding sites by the 6-hydroxydopamine. However, since 6-hydroxydopamine is very similar to noradrenaline in its chemical properties (Senoh & Witkop, 1959) it was possible that the 6-hydroxydopamine or some metabolite replaces the noradrenaline in the heart tissue and is then bound there, preventing the resynthesis or replacement of the lost noradrenaline.

In order to investigate this question a method was devised to isolate and measure the 6-hydroxydopamine content of tissue samples, and so to see whether its persistence in the tissues could explain its action on the storage of noradrenaline. The functional state of the nervi accelerantes was tested at different time intervals after the administration of 6-hydroxydopamine, and the effect of the drug on the release of catechol amines from the adrenal medulla was examined.

## METHODS

Adult male and female mice, young female guinea-pigs, and kittens and puppies of either sex were used in these experiments. 6-Hydroxydopamine was injected intraperitoneally or intravenously as the hydrobromide; all doses of 6-hydroxydopamine and other amines are expressed in terms of the bases.

*Cardiac nerve stimulation.* Kittens were anaesthetized with ether followed by intravenous chloralose (80 mg/kg) and the chest was opened under artificial ventilation. In some kittens the adrenal glands were removed before opening the chest; in many, the blood pressure was measured by a mercury manometer connected to the carotid artery. The right stellate ganglion was exposed and the strand of postganglionic nerves going to the heart was placed on shielded electrodes. Rectangular pulses of 1 msec duration from a Grass SD5 stimulator were used at a frequency of 15 shocks/sec; the voltage applied was usually 20 V. The heart rate was measured from the electrocardiogram recorded on a Grass Polygraph.

*Perfusion of adrenal glands.* Adrenal glands of puppies were perfused by gravity at 35° C with Locke solution saturated with 95% oxygen and 5% carbon dioxide. The perfusion cannula was tied into the superior mesenteric artery. The dissection was carried out as previously described (Vogt, 1951), but the isolated segments of aorta and vena cava to which the left adrenal gland was attached were excised from the body together with the adrenal gland and placed on a perforated platform resting in a funnel. The

* Beit Memorial Research Fellow. Present address: Wellcome Research Institute, University of Otago Medical School, Dunedin, New Zealand.

6-hydroxydopamine was freshly dissolved in bicarbonate-free Locke solution to make a 0.2% solution. Of this solution, 0.2 ml. was slowly infused by means of a precision syringe fitted to a narrow polyethylene tube which opened into the tip of the arterial cannula. In this way it was possible to give the drug without addition of reducing chemicals to prevent its destruction.

*Catechol amine estimations*. Mice were stunned and bled to death. Guinea-pigs and kittens were anaesthetized with chloroform or chloralose and bled to death. The hearts and other tissues were removed, blotted dry and stored for less than 3 hr at −16° C. In the kitten experiments only the atria of the heart were used; mouse hearts were handled as groups of four to six. Heart tissues were frozen in liquid nitrogen, crushed while frozen and then homogenized with twice their weight of 0.1 N-hydrochloric acid; brain tissues were homogenized directly in 0.1 N-hydrochloric acid. The proteins were precipitated with perchloric

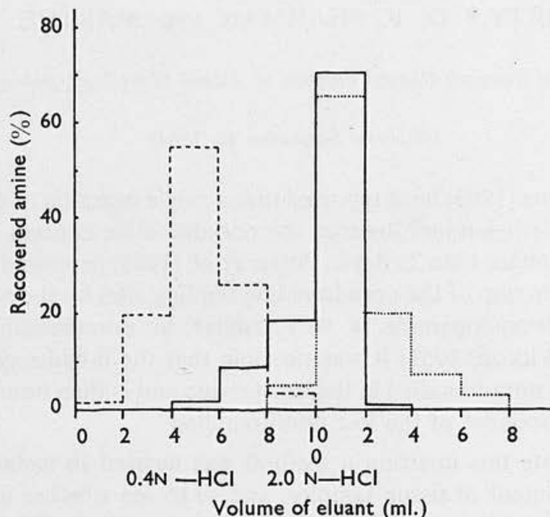


Fig. 1. Elution pattern of noradrenaline (– – – –), 6-hydroxydopamine (——) and dopamine (. . . .) from a Dowex 50 X-8 ion-exchange resin column (2.5×0.4 cm) using 0.4 N- and 2 N-hydrochloric acid.

acid, and the amines were extracted by passing the deproteinized extract, after adjusting to *p*H 4 with potassium carbonate, through a column of Dowex 50 X-8 ion-exchange resin (2.5×0.4 cm) (Bertler, Carlsson & Rosengren, 1958). The column was washed with 4 to 6 ml. of water. Noradrenaline was eluted with 10 ml. of 0.4 N-hydrochloric acid, and other catechol amines were removed with 8 ml. of 2 N-hydrochloric acid (Fig. 1). When separation of the catechol amines was to be carried out by paper chromatography they were eluted from the column with 8 ml. of 2 N-hydrochloric acid.

Noradrenaline in the first eluate was determined fluorimetrically after oxidation by ferricyanide (Euler & Lishajko, 1961; for details see Sharman, Vanov & Vogt, 1962). The catechol amines in the second eluate were estimated fluorimetrically after acetylation (Laverty & Sharman, 1964) by condensation with ethylenediamine; 4 ml. of column eluate, after treatment with 0.3 ml. of acetic anhydride followed by excess of sodium bicarbonate, were heated for 20 min at 65° C with 0.5 ml. of a mixture of 3 vols of ethylenediamine and 2 vols of 2 N-hydrochloric acid. The reaction mixture was cooled, saturated with sodium chloride and extracted with 3 ml. of isobutanol. The fluorescence of the organic phase was measured.

The quantitative measurements of the catechol amines were carried out on a Locarte filter fluorimeter. For noradrenaline the filters used were primary, Chance OX1, and secondary, Ilford Bright Spectrum 625. For catechol amines after ethylenediamine condensation the primary filter was a combination of Corning 3389 and Corning 5113 (half standard thickness); the secondary filters were Ilford Bright Spectrum 623 and Ilford Bright Spectrum 625 (see later).

For further purification by paper chromatography, the column eluate was treated with acetic anhydride and sodium bicarbonate and the total acetylated amines were extracted from aqueous solution with

dichloromethane; the dichloromethane extract was evaporated almost to dryness and the concentrated extract was applied to alkali-washed Whatman No. 50 paper (Sharman, 1963). The developing solvent was a modification of the system used for steroids (Eberlein & Bongiovanni, 1955) and consisted of the organic phase from a mixture of petroleum ether (boiling point, 80 to 100° C), water and *tert.*-butanol (8 : 8 : 3). The paper was equilibrated in the presence of the aqueous phase for 12 to 18 hr and a descending chromatogram was developed for 24 to 48 hr. Authentic catechol amines were acetylated and run on each chromatogram and visualized by heating the paper after spraying with a five-fold dilution of the ethylenediamine–hydrochloric acid mixture described above. The chromatograms of tissue extracts were cut into 1.5-cm lengths and each piece was eluted with 4 ml. of water. The catechol amines were estimated fluorimetrically after condensation with ethylenediamine as described above.

In the experiments on kittens and guinea-pigs, the small quantities of noradrenaline present in the superior cervical and stellate ganglia were measured biologically on the pithed rat, following separation of the amines by paper chromatography using a phenol–hydrochloric acid solvent (Vogt, 1954). Because 6-hydroxydopamine and noradrenaline are " isographic " (Senoh & Witkop, 1959), any 6-hydroxydopamine present in the extract would be found on the chromatogram with the noradrenaline. However, since the pressor activity of noradrenaline is about 3,000-times that of 6-hydroxydopamine, the amount which might conceivably have been present in the tissue extract was far below threshold dose in the bioassay.

*Separation and estimation of 6-hydroxydopamine.* 6-Hydroxydopamine gave no fluorescent product when subjected to the ferricyanide oxidation method used for noradrenaline and adrenaline; 1 $\mu$g of 6-hydroxydopamine gave less fluorescence than 1 ng of noradrenaline. Using the iodine oxidation technique of Carlsson & Lindqvist (1962), 1 $\mu$g of 6-hydroxydopamine gave a fluorescence equivalent to less than that of 30 ng of dopamine but, when condensed with ethylenediamine, it formed a fluorescent product of similar intensity and wavelength to that of dopamine.

After acetylation, condensation of catechol amines with ethylene diamine gave a greater fluorescence intensity than before (Laverty & Sharman, 1964). The same held for 6-hydroxydopamine. In isobutanol the acetylated 6-hydroxydopamine derivative had similar fluorescence characteristics to the acetylated noradrenaline derivative, but different ones from those of the acetylated dopamine derivative (Laverty & Sharman, 1965).

Separation of 6-hydroxydopamine from other catechol amines was attempted using both column and paper chromatography. The elution pattern of 6-hydroxydopamine from Dowex 50 X-8 resin using hydrochloric acid is compared in Fig. 1 with those of noradrenaline and dopamine. It will be seen that the 6-hydroxydopamine is eluted predominantly in the dopamine eluate (the 2 N-hydrochloric acid eluate); any 6-hydroxydopamine eluted with the noradrenaline in the 0.4 N-hydrochloric acid eluate did not interfere with the estimation of noradrenaline by the ferricyanide-oxidation technique. The total recovery of amine was for noradrenaline 89% from 0.2 $\mu$g, for 6-hydroxydopamine 90% from 5 $\mu$g, and for dopamine 82% from 2 $\mu$g.

On paper, 6-hydroxydopamine is " isographic " with noradrenaline (Senoh & Witkop, 1959). In order to separate the two amines, the acetyl derivatives were formed and run in a descending paper chromatogram with the solvent system described above for catechol amines.

In this solvent system, acetylated noradrenaline, adrenaline, 6-hydroxydopamine and dopamine were separated (Fig. 2), though there was, in some experiments, a tendency for the tail of the dopamine spot to overlap the 6-hydroxydopamine spot. The mean recovery of amines after acetylation, extraction with dichloromethane and chromatography was for noradrenaline 71% from 0.2 to 1.0 $\mu$g, for adrenaline 68% from 1 $\mu$g, for 6-hydroxydopamine 49% from 1 to 5 $\mu$g, and for dopamine 63% from 0.2 to 1.0 $\mu$g.

Since both column and paper chromatography separated the 6-hydroxydopamine and dopamine from the noradrenaline, a method was required for the measurement of 6-hydroxydopamine in the presence of dopamine. After condensation with ethylenediamine the fluorescence spectra of acetylated dopamine and 6-hydroxydopamine were sufficiently different to make a differential estimation feasible. With an Ilford Bright Spectrum 623 filter, the fluorescence intensity per $\mu$g of dopamine was approximately twice that of 6-hydroxydopamine whereas, using an Ilford Bright Spectrum 625 filter, it was approximately six-times that of 6-hydroxydopamine. Using these filter sets it was possible to determine with reasonable precision quite small amounts of one amine in the presence of the other (Table 1).

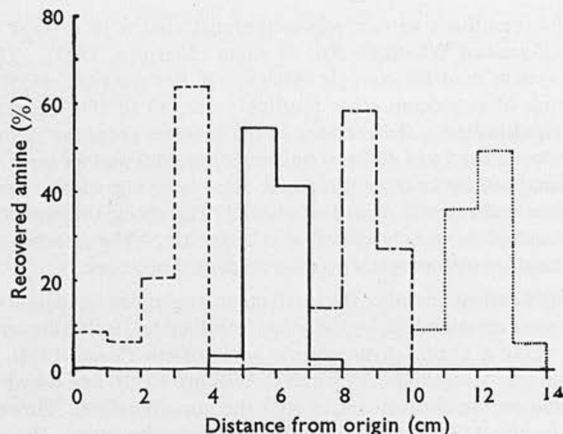


Fig. 2. Separation of the acetylated derivatives of noradrenaline (- - - -), adrenaline (——), 6-hydroxydopamine (- . - . -) and dopamine (. . . .) on Whatman No. 50 paper using a petroleum ether, water and *tert*.-butanol (8 : 8 : 3) solvent system. The chromatogram was developed downwards for 24 hr, and then cut into 1-cm strips.

TABLE 1

THE ESTIMATION OF DOPAMINE AND 6-HYDROXYDOPAMINE IN MIXTURES

The amines were acetylated, condensed with ethylenediamine and the amounts measured fluorimetrically using two different fluorescence wavelengths

| Dopamine (ng) | | 6-Hydroxydopamine (ng) | |
|---|---|---|---|
| Added | Measured | Added | Measured |
| *A. Sample in 4 ml. of water* | | | |
| 200 | 220 | 2 | −33 |
| 200 | 213 | 5 | −22 |
| 200 | 196 | 10 | 7 |
| 200 | 201 | 50 | 42 |
| 100 | 98 | 100 | 135 |
| 100 | 96 | 100 | 114 |
| 50 | 47 | 200 | 185 |
| 50 | 52 | 500 | 476 |
| 25 | 22 | 500 | 462 |
| 10 | 10 | 500 | 419 |
| 100 | 96 | 500 | 476 |
| *B. Sample added to 4 ml. of eluate* | | | |
| 100 | 88 | 300 | 324 |
| 100 | 92 | 300 | 290 |
| 100 | 90 | 300 | 302 |
| 100 | 93 | 300 | 302 |
| 100 | 106 | 300 | 323 |
| 100 | 102 | 300 | 316 |
| 100 | 99 | 300 | 332 |

## RESULTS

*Effect of 6-hydroxydopamine on catechol amine concentrations in mouse tissues.* In a preliminary experiment on two groups of three mice each, it was found that 16 hr after an injection of 6-hydroxydopamine (6.7 mg/kg, intraperitoneally) the noradrenaline content of the hearts was reduced from 0.42 to 0.16 $\mu$g/g; there was no observable change in the

noradrenaline content of the brain, spleen or lung, nor in the dopamine content of any of these four tissues. In all further experiments on mice, only the noradrenaline and 6-hydroxydopamine contents of the hearts were measured.

Two strains of mice were used, one for the experiments involving intraperitoneal injections of 6-hydroxydopamine and dopamine, the other for the intravenous experiments. They were found to differ in the initial noradrenaline concentration of their hearts. The results are summarized in Fig. 3,*a*. It will be seen that the noradrenaline content of the

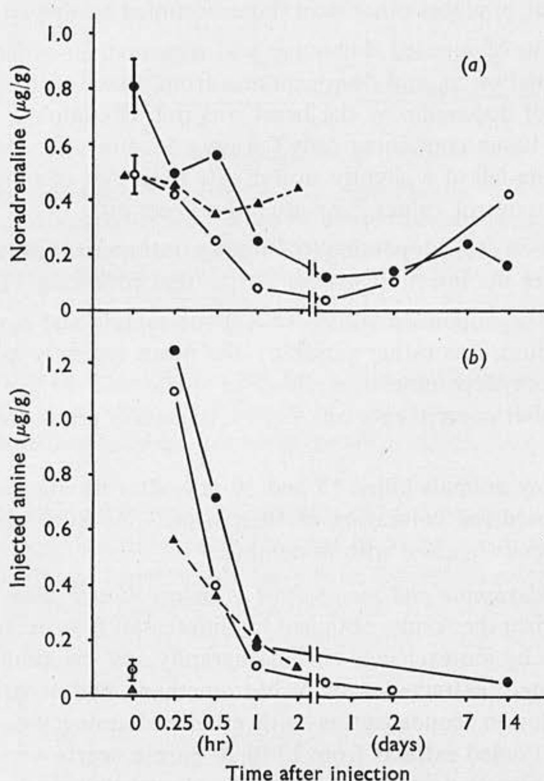


Fig. 3. The concentration in heart tissue of noradrenaline (*a*) and 6-hydroxydopamine or dopamine (*b*) following the injection of 10 mg/kg of 6-hydroxydopamine (○, intraperitoneally; ●, intravenously) or 10 mg/kg of dopamine (▲, intraperitoneally). The means and standard deviations given for control values (zero time) were obtained using six groups of five mice each; other points represent the means from two or more such groups.

mouse heart fell rapidly following the injection of 6-hydroxydopamine (10 mg/kg) by either route, the fall being almost complete at 1 hr after the injection. The level of noradrenaline recovered very slowly, and was still quite low, particularly in the intravenously injected group, as long as 14 days after the injection. However, with a dose of 6.7 mg/kg intraperitoneally, the noradrenaline content of the heart was back to 70% of its initial value at 17 hr. After injection of dopamine (10 mg/kg), used for comparison, there was no significant change in the noradrenaline content of the heart.

*Estimation of 6-hydroxydopamine concentrations in mouse heart by differential fluorimetry.* After injection of 6-hydroxydopamine its concentration in the heart rose to over 1 μg/g 15 min after the injection, and then fell steeply, being almost back to control values at 1 hr (Fig. 3,*b*). The values of 6-hydroxydopamine given for control hearts probably represent a background fluorescence from undetermined materials rather than an actual tissue content of 6-hydroxydopamine, because no 6-hydroxydopamine could be detected in control hearts following further purification by paper chromatography. Small amounts of materials having fluorescence characteristics similar to those of 6-hydroxydopamine were detected on the paper in places other than those occupied by known catechol amines.

The tissue concentration of injected dopamine was measured in order to compare the time course of its accumulation in, and disappearance from, tissue with that of 6-hydroxydopamine. The uptake of dopamine by the heart was not as complete as the uptake of 6-hydroxydopamine, the tissue containing only 0.6 μg/g 15 min after the injection. The tissue content of dopamine fell at a slightly slower rate than that of 6-hydroxydopamine, and had not returned to control values 2 hr after the injection.

With a lower dose of 6-hydroxydopamine (6.7 mg/kg, intraperitoneally), the amount in the heart 1 and 17 hr after the injection was similar to that following 10 mg/kg.

The recovery of 6-hydroxydopamine, added to a tissue sample and carried through the column extraction procedure, was rather variable; the mean recovery in five experiments in which 1 μg of 6-hydroxydopamine was added to tissue was 60%. The recovery of dopamine (0.5 μg) in similar experiments was 73%. All figures given are uncorrected for recovery.

It was noticed that many animals killed 15 and 30 min after an injection of 6-hydroxydopamine had a pronounced red coloration of their urine. No such colour was seen in control animals or in animals injected with dopamine.

*Detection of 6-hydroxydopamine and metabolites in mouse hearts after paper chromatography.* In order to confirm the results obtained by differential fluorimetry, treated hearts were extracted for amines by ion-exchange chromatography and the amines in the column eluates were then acetylated, extracted with dichloromethane and separated on a paper chromatogram. After elution, condensation with ethylenediamine was carried out and the fluorescence plotted. Pooled extracts from 13 to 20 mouse hearts were used. An equal number of hearts from uninjected controls was analysed simultaneously: in none of the three experiments was there any 6-hydroxydopamine found. The amount detectable by the present technique was less than 30 ng; allowing for an approximate total recovery of 30%, this would place an upper limit on the tissue concentration of 6-hydroxydopamine in mouse hearts at 0.04 μg/g. It was possible in these experiments to detect the presence of adrenaline and dopamine in some extracts of mouse hearts; the amounts were too small to be measured accurately but were of the order of 0.03 μg/g for adrenaline and 0.01 μg/g for dopamine.

The hearts of mice which had been treated with 6-hydroxydopamine 15 min and 16 hr before being killed were extracted and analysed. At 15 min after injection of 6-hydroxydopamine, at a time when the noradrenaline content was only slightly lower than that of the control hearts, there was a fluorescence peak on the chromatogram (Fig. 4) corresponding to approximately 1.1 μg of 6-hydroxydopamine. This amount represents a tissue

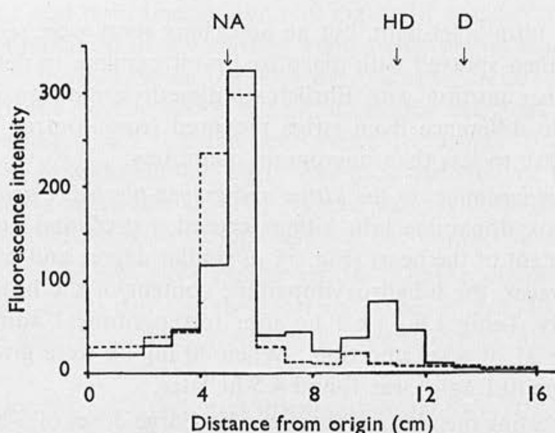


Fig. 4. Fluorescence scan of chromatograms from extracts of hearts. (- - - -) Control mice, (——) mice killed 15 min after intraperitoneal injection of 6-hydroxydopamine (10 mg/kg). The extracts were from approximately 3 g of heart and chromatograms were developed downwards for 48 hr in a petroleum ether, water and *tert.*-butanol (8 : 8 : 3) solvent. Control spots of known catechol amines run in parallel were found at, for noradrenaline (NA) 5 to 6 cm, 6-hydroxydopamine (HD) 11 to 12 cm, and dopamine (D) 14 cm from the origin. The fluorescence is measured in arbitrary units.

concentration similar to that obtained by direct differential fluorimetry. The chromatogram from mouse hearts 16 hr after the injection of 6-hydroxydopamine showed a pronounced fall in the noradrenaline content from the control values, but no detectable 6-hydroxydopamine peak.

Since, therefore, residual 6-hydroxydopamine did not appear to replace the noradrenaline of the heart, two experiments were carried out in which a search was made for any other phenolic or indolic bases which might have been formed as metabolites of 6-hydroxydopamine. Groups of about twenty mice were killed 3 hr after intraperitoneal injection of 6-hydroxydopamine (10 mg/kg), and chromatograms of the heart extracts run as before.

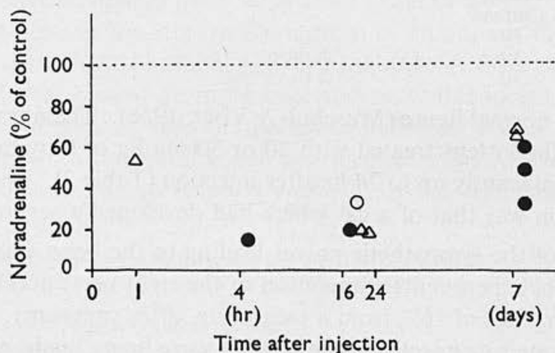


Fig. 5. The depletion of the noradrenaline from kitten hearts by 6-hydroxydopamine. The doses used were 10 mg/kg intraperitoneally (△), 20 mg/kg intravenously (○), and 30 mg/kg intravenously (●). The results are calculated in terms of the percentage of the noradrenaline content of hearts from litter-mate controls; the noradrenaline content (mean and standard deviation) of the hearts of eight kittens was $0.90 \pm 0.30$ $\mu$g/g.

They were scanned with ultraviolet light, but no absorbing spots were seen. A portion of the chromatogram was then sprayed with diazotized *p*-nitroaniline to detect any phenolic compounds, and the other portion with Ehrlich's *p*-dimethylaminobenzaldehyde reagent to look for indoles. No difference from strips prepared from control hearts was seen; these tests are not sensitive to less than microgram quantities.

*The effect of* 6-*hydroxydopamine on the kitten and guinea-pig heart and on other tissues.* The injection of 6-hydroxydopamine into kittens caused a profound and prolonged fall in the noradrenaline content of the heart (Fig. 5), of similar degree and time-course as that observed in mice. However, the 6-hydroxydopamine content of the hearts of the treated animals fell more slowly, being 1.6 μg/g 1 hr after intraperitoneal administration of 10 mg/kg, but undetectable 21 hr after injection. When 30 mg/kg were given intravenously, some 6-hydroxydopamine (0.1 μg/g) was found 4.5 hr later.

Relaxation of the nictitating membrane was seen when large doses of 6-hydroxydopamine had been injected 6 hr or more previously; therefore the noradrenaline content of the superior cervical ganglia was estimated in these experiments. No significant changes were seen (Table 2). Neither was there any change in the noradrenaline content of stellate ganglion of the cat examined 18 hr after the highest dose; it was 5.1 μg/g which is even

TABLE 2

THE EFFECT OF 6-HYDROXYDOPAMINE ON THE NORADRENALINE CONTENT OF NERVOUS TISSUES IN KITTENS

Injections were intravenous

| | | | Noradrenaline content (μg/g) | |
|---|---|---|---|---|
| Litter No. | Dose (mg/kg) | Duration | Superior cervical ganglia | Hypothalamus |
| 1 | Control | | 3·6 | 2·3 |
| | 30 | 4·5 hr | 3·1 | 0·7 |
| | 30 | 18 hr | 3·2 | 1·1 |
| 2 | Control | | 4·4 | 1·6 |
| | 20 | 20 hr | 4·4 | 0·6 |
| 3 | Control | | — | 1·3 |
| | Control | | — | 1·9 |
| | 30 | 8 days | — | 0·7 |
| | 30 | 8 days | — | 1·2 |
| | 30 | 8 days | — | 1·3 |

higher than the average normal figure (Muscholl & Vogt, 1958). The noradrenaline content of the hypothalami of the kittens treated with 20 or 30 mg/kg of 6-hydroxydopamine was found to be lowered significantly up to 24 hr after injection (Table 2). The single low figure 8 days after the injection was that of a cat which had developed a severe infection.

The functional state of the sympathetic nerves leading to the heart was tested in a series of kittens. In six control experiments stimulation of the right nervi accelerantes caused an average increase of heart rate of 16% from a mean rate, after vagotomy, of 235 beats/min. When 6-hydroxydopamine was injected while records were being made, acceleration of the heart by stimulation of the cardiac nerves was immediately abolished. However, in one cat, which had not been adrenalectomized, the heart rate rose spontaneously by 25% (to 342) in the course of the next 90 min. A smaller rise of 10% occurred in an adrenalectomized cat following the intravenous injection of 6-hydroxydopamine; since it only developed slowly, it was not responsible for the immediate failure of nerve stimulation to increase heart rate.

In four kittens that had been injected with 10 mg/kg of 6-hydroxydopamine either 1 or 7 days previously, stimulation of the cardiac sympathetic nerves caused a mean increase in heart rate of 11%. However, when the dose was increased to 20 to 30 mg/kg, even 8 days later stimulation of the cardiac nerves did not accelerate the heart or raise the blood pressure. In one experiment in which the heart failed to respond to nerve stimulation after treatment with 6-hydroxydopamine, an intravenous injection of noradrenaline caused a prolonged increase in heart rate of 20%. There was no correlation between the ability of the heart to respond to nerve stimulation and the noradrenaline content of the heart.

Experiments in guinea-pigs gave similar results to those obtained in mice and kittens, in that 6-hydroxydopamine depleted the heart and spleen of noradrenaline without affecting the noradrenaline content of the sympathetic ganglia (Table 3). Yet comparison with

TABLE 3

EFFECTS OF 6-HYDROXYDOPAMINE IN THE GUINEA-PIG

Injections were intraperitoneal in the first, and intravenous in the other experiments

| Dose (mg/kg) | Duration (hr) | Noradrenaline content: Heart (μg/g) | Stellate ganglia (μg/g) | Superior cervical ganglia (μg/g) | Spleen (μg/spleen) | 6-Hydroxy-dopamine content of heart (μg/g) |
|---|---|---|---|---|---|---|
| Control | | 1·5 | 2·8 | 6·1 | 0·38 | 0·01 |
| | | 1·5 | 2·5 | 4·6 | 0·50 | 0·00 |
| | | 1·7 | 1·2 | 4·3 | 0·42 | |
| | | | 2·8 | 5·0 | | |
| | | | 3·9 | 7·0 | | |
| 10 | 16 | 1·0 | 3·4 | 5·5 | 0·21 | 0·25 |
| 10 | 2 | 0·34 | 3·4 | 3·8 | 0·10 | 1·10 |
| 15 | 2·5 | 0·35 | 2·2 | 3·4 | 0·12 | 0·50 |

Fig. 3 shows certain differences: 16 hr following intraperitoneal injection of 10 mg/kg of 6-hydroxydopamine, only a small fall in the noradrenaline content of the heart had occurred in the guinea-pig, and the 6-hydroxydopamine did not disappear from the heart as rapidly as in the mouse.

*The release of catechol amines from the adrenal gland by 6-hydroxydopamine.* The effect of 6-hydroxydopamine on the storage mechanism of adrenomedullary amines was investigated in the isolated perfused adrenal gland of the dog. A dose of 0.27 mg infused over a period of 75 to 100 sec caused an immediate and reversible increase in the release of both noradrenaline and adrenaline into the perfusate collected over a 2-min period (Table 4). There was no damage to the gland which was seen to revert to a lower secretion rate after the end of the infusion and to respond subsequently to stimulation by tyramine hydro-

TABLE 4

MEDULLARY AMINES RELEASED BY THE ISOLATED PERFUSED ADRENAL GLAND OF THE DOG IN RESPONSE TO AN INFUSION OF 0·27 mg OF 6-HYDROXYDOPAMINE

| Adrenal weight (g) | Noradrenaline released: Before infusion (ng/min) | During infusion (ng/min) | Increase (%) | Adrenaline released: Before infusion (ng/min) | During infusion (ng/min) | Increase (%) |
|---|---|---|---|---|---|---|
| 0·25 | 53 | 90 | 70 | 300 | 500 | 67 |
| 0·28 | 45 | 138 | 206 | 300 | 500 | 67 |
| 0·41 | 50 | 120 | 140 | 450 | 1,100 | 144 |

chloride, prenylamine or dimethylphenylpiperazinium iodide. The flow through the adrenal gland was not affected by the drug. The percentage methylation (adrenaline as percentage of the total amines) was not or very little changed during stimulation by the drug.

## DISCUSSION

The present experiments confirm those of Porter *et al.* (1963) concerning the prolonged action of 6-hydroxydopamine on the noradrenaline content of the mouse heart. The action of this drug has a rapid onset, but even after a single injection the effect lasts for more than 1 week in both mice and kittens. We have also shown in guinea-pigs and kittens that 6-hydroxydopamine has no appreciable action on the noradrenaline content of sympathetic ganglia; our findings differ from those in dogs (Stone, Stavorski, Ludden, Wenger, Ross, Totaro & Porter, 1963) with 6-aminodopamine in that a high dose of 6-hydroxydopamine (20 to 30 mg/kg) depleted the hypothalamic noradrenaline in cats. Thus 6-hydroxydopamine appears to have selective effects on different parts of the peripheral and central nervous system.

6-Hydroxydopamine not only lowers the noradrenaline content of heart tissue but also releases catechol amines from the dog adrenal medulla (Table 4). This effect explains the rapid increase in heart rate seen in anaesthetized kittens immediately following an intravenous injection of 6-hydroxydopamine, an effect which was reduced but not abolished by adrenalectomy. In dogs, also, 6-hydroxydopamine caused a prolonged increase in heart rate (Stone *et al.*, 1963).

It has been suggested that 6-hydroxydopamine might be a naturally occurring amine, at least in the rat (Senoh, Creveling, Udenfriend & Witkop, 1959). Our experiments using a paper chromatographic separation failed to show any 6-hydroxydopamine in untreated mouse hearts. This does not, however, eliminate the possibility that quantities not detectable by our methods may occur, particularly in tissues rich in dopamine.

A number of phenylethylamine derivatives are thought to lower noradrenaline concentration in tissues by direct replacement of the noradrenaline; tyramine (Schümann & Philippu, 1962), $\alpha$-methylnoradrenaline (Maître & Staehelin, 1963) and dopamine (Harrison, Levitt & Udenfriend, 1963) have all been shown to replace noradrenaline stoichiometrically. However, it is obvious from our experiments that, except during the first hour after injection, there is not sufficient 6-hydroxydopamine present in the tissue of mice and kittens to replace the lost noradrenaline. Since 6-hydroxydopamine disappeared so rapidly from the tissue, it was possible that a metabolic product was formed which was responsible for its action. However, no basic metabolite could be detected in extracts from treated mouse hearts. This may have been due to the metabolites being acidic or neutral, chemically unstable and thus lost during extraction, or being present only in small quantities. Such a metabolite would be unlikely to act as a replacement for a considerable quantity of noradrenaline for periods of longer than a week. 6-Hydroxydopamine does not affect uptake of noradrenaline into the rat heart so that it appears not to act in the tissue like a sympathomimetic amine (Iversen, 1964).

It is possible that only small amounts of 6-hydroxydopamine need be present to release, and to maintain the depletion of, stored noradrenaline. Such small amounts may persist in

tissues, as do small amounts of reserpine (Maggiolo & Haley, 1964) and guanethidine (Bisson & Muscholl, 1962). Some other mode of action than replacement must then be postulated.

The fact that the noradrenaline content of heart remains depressed after a single injection of 6-hydroxydopamine for many days has been interpreted (Porter *et al.*, 1963) as permanent damage to the binding sites in the heart. This view is supported by the demonstration of rapid disappearance of the compound from the tissues and our inability to find any persisting metabolite. 6-Hydroxydopamine is a strong reducing agent, very unstable and forming red oxidation products readily. It is, therefore, not unlikely that its metabolic path is totally different from that of sympathomimetic amines and that it causes irreversible damage to receptor sites by virtue of its great chemical reactivity.

In addition to damage to storage mechanisms in the heart, there is functional failure of transmission at sympathetic endings of several organs. In the heart, this occurs immediately after an intravenous injection, when depletion has not yet had time to develop, and is still present a week later, when the noradrenaline content of the heart is reduced by about 50% at most. Furthermore, relaxation of the nictitating membrane was seen at periods ranging from 6 hr to 3 days after an injection of 6-hydroxydopamine (30 mg/kg); in the earlier part of the experiment, it was probably overshadowed by release of amines from the adrenal medulla. Yet the noradrenaline content of the superior cervical ganglia was normal, as was, indeed, that of the stellate ganglion at a time when impulse transmission to the heart was abolished. A similar lack of correlation between block of transmission and depletion of tissue noradrenaline was seen in the dog by Stone *et al.* (1963). It is possible to explain these observations on a multiple-pool concept of storage of tissue noradrenaline, by assuming a complete depletion of the readily available noradrenaline without necessarily a complete depletion of more firmly bound stores. However, they could be explained equally well by assuming that 6-hydroxydopamine has a bretylium-like action on adrenergic nerves. This hypothesis would be more consistent with the observations that the noradrenaline content of the stellate and other ganglia, from which the adrenergic neurones originate, is normal when the peripheral nerve ending is no longer releasing noradrenaline.

## SUMMARY

1. A single injection of 6-hydroxydopamine (10 to 30 mg/kg) lowered the noradrenaline content of mouse, kitten and guinea-pig hearts for a period ranging from less than 1 hr to more than 1 week.

2. Methods for the isolation and fluorimetric estimation of 6-hydroxydopamine in tissue were developed. Ion-exchange chromatography, alone or followed by paper chromatography, was used for separation of 6-hydroxydopamine from catechol amines. The 6-hydroxydopamine did not remain in the heart of the mouse for more than 1 hr after the injection.

3. No bases which might have been metabolites of 6-hydroxydopamine were detected in the heart 3 hr after injection of the drug.

4. In kittens and guinea-pigs, 6-hydroxydopamine did not lower the noradrenaline content of the superior cervical or stellate ganglia; large doses (20 to 30 mg/kg) reduced the hypothalamic noradrenaline in kittens.

5. Large doses of 6-hydroxydopamine in kittens abolished the increase in heart rate due to stimulation of the cardiac postganglionic sympathetic nerves; smaller doses (10 mg/kg) reduced this response. No correlation was found between the response to nerve stimulation and the noradrenaline content of the heart.

6. 6-Hydroxydopamine released noradrenaline and adrenaline from the isolated perfused adrenal gland of the dog.

7. The prolonged and specific depletion of noradrenaline from peripheral stores is not due to replacement by 6-hydroxydopamine. Other possible mechanisms of action are discussed.

We are grateful to J. E. McEwen, F.I.S.T., for his skilful help in the noradrenaline estimations. We would like to thank Dr C. S. Miller (Merck Sharp & Dohme Research Laboratories Inc.) for a gift of 6-hydroxydopamine.

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## The noradrenaline content of the caudate nucleus of the rabbit

(*Received* 14 *September* 1964)

IN A recent paper (MATSUOKO, YOSHIDA and IMAIZUMI, 1964) it was stated that there is a high noradrenaline concentration (0·85 μg/g) in the rabbit caudate nucleus. In all species previously examined the caudate nucleus has been found to contain high concentrations of dopamine, but hardly any noradrenaline. In order to ascertain whether there existed an interesting species difference between the rabbit and other mammals, we estimated the noradrenaline in the caudate nucleus of adult rabbits (weight 2·5–3 kg) by three different methods: (1) a bioassay on the pithed rat's blood pressure following paper chromatographic separation of the catecholamines (VOGT, 1954), (2) a fluorimetric assay by a method comparable to that used by MATSUOKO *et al.* (1964) consisting of adsorption of the amines on a column of resin followed by oxidation of the eluate by ferricyanide to form a fluorescent compound (VON EULER and LISHAJKO, 1961), and (3) a fluorimetric method in which the amines in the eluate from the column were acetylated, chromatographed on paper and the fluorimetry was carried out on the eluate from the paper after condensation with ethylene diamine (LAVERTY and SHARMAN, 1965). Table 1 shows the results obtained with the three methods; the results of method 3, in which the losses are greater, have been corrected to 70 per cent recovery which is the recovery achieved in methods 1 and 2.

TABLE 1.—NORADRENALINE CONTENT ESTIMATED BY DIFFERENT METHODS IN THE CAUDATE NUCLEUS OF THE RABBIT

| | μg/g fresh tissue | | |
|---|---|---|---|
| | Method 1 | Method 2 | Method 3 |
| Experiment 1 | <0·025 | 0·33 | 0·045 |
| Experiment 2 | 0·017 | 0·18 | 0·027 |

It is evident from the Table that method 2 gives much higher figures than methods 1 and 3. A likely explanation is that there exists, in the rabbit caudate nucleus, a substance which is not noradrenaline but which is adsorbed on strong cation exchange resins and gives a fluorescent compound with ferricyanide. It is probable that MATSUOKO *et al.* (1964) encountered such a phenomenon in their extracts. Recently a similar difficulty has arisen in our laboratory in assays of noradrenaline in the rat hypothalamus; the figures obtained were 50 per cent higher with method 2 than with methods 1 and 3.

The discrepancies might also be due to the presence in the tissues of fluorescent material which loses its fluorescence on the addition of ferricyanide and alkali, and causes the "faded blanks", used for reference, to be too low.

*Agricultural Research Council*
*Institute of Animal Physiology*
*Babraham*
*Cambridge*

D. F. SHARMAN
MARTHE VOGT

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## Gas chromatographic evidence for the presence of glycol metabolites of catecholamines in brain tissue

By D. F. Sharman. *A.R.C. Institute of Animal Physiology, Babraham, Cambridge*

It has been shown by Rutledge & Jonason (1967) that rabbit brain slices can metabolize noradrenaline *in vitro* to 3,4-dihydroxyphenylethyleneglycol (DOPEG) and 4-hydroxy-3-methoxyphenylethyleneglycol

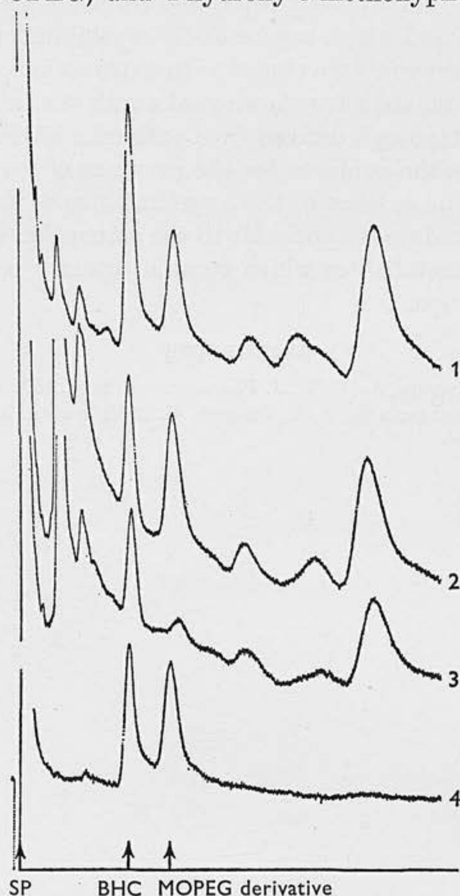


Fig. 1. Gas chromatogram record of: 1. Extract of rabbit hypothalamus. 2. Extract of rabbit cerebral cortex to which authentic 4-hydroxy-3-methoxyphenylethyleneglycol (MOPEG) had been added. 3. Extract of rabbit cerebral cortex. 4. Authentic MOPEG derivative. SP, solvent peak. BHC, Benzene hexachloride used as a reference compound. The gas chromatogram was developed on a 180 cm column of 3·8 % S.E. 30 on silanized Diatoport S (60–80 mesh). Column temperature 135° C; detector temperature 180° C; pulse interval 50 μsec; carrier gas argon: 5 % methane; gas flow 30 ml./min at 1·7 kg/cm².

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(MOPEG). Schanberg, Schildkraut, Breese & Kopin (1968) have reported that the ethereal sulphate conjugate of MOPEG was present in normal rat brain but were unable to detect the free compound.

DOPEG and MOPEG can be converted to heptafluorobutyric esters which can be separated by gas chromatography and measured by the electron capture detector. It was found that acetylation of the phenolic hydroxyl groups, before the esterification of the alcoholic hydroxy groups with heptafluorobutyric anhydride, yielded compounds with MOPEG and DOPEG which were more easily separated from the solvent peak of the gas chromatogram and which can be easily crystallized to form reference standards. Chromatograms developed with extracts prepared from rabbit and mouse hypothalamus have shown peaks with the same relative retention time as the compounds derived from authentic MOPEG and DOPEG. Figure 1 illustrates the evidence for the presence of very small amounts of free MOPEG in an extract of the hypothalamus of the rabbit.

The general procedure is applicable to the estimation of those catecholamines and their metabolites which contain primary or secondary alcoholic hydroxyl groups.

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Glycol metabolites of noradrenaline in brain tissue.

By

D. F. Sharman.

Agricultural Research Council

Institute of Animal Physiology,

Babraham, Cambridge.

Summary

1. A gas chromatographic method using electron capture detection is described for the estimation of those catecholamines and their metabolites which possess one or two free alcoholic hydroxyl groups. It is based on acetylation of phenolic hydroxyl and primary and secondary amino groups in aqueous solution, extraction into a water immiscible solvent and esterification of alcoholic hydroxyl groups with heptafluorobutyric anhydride.

2. The method has been successfully applied to the detection and estimation of free 4-hydroxy-3-methoxyphenyl-ethyleneglycol (MOPEG) and free 3,4-dihydroxyphenyl-ethyleneglycol (DOPEG) in hypothalamic brain tissue.

3. Although it was possible to measure noradrenaline and normetanephrine by the same method in pure solution, crude tissue extracts could not be used, despite their suitability for estimating the glycol metabolites.

## Introduction

In 1963, Mannarino, Kirshner and Nashold showed that when DL- $\beta$-$^{14}C$ - noradrenaline was injected into the lateral cerebral ventricle of the cat, it was metabolised in the brain to 4-hydroxy-3-methoxyphenylethyleneglycol (MOPEG) and 3,4-dihydroxyphenylethyleneglycol (DOPEG). Glowinski, Kopin and Axelrod (1965) injected tritiated noradrenaline into the cerebral ventricles of the rat and also found that the major part of the deaminated metabolites formed in the brain consisted of MOPEG. The presence of a sulphate conjugate of MOPEG in normal rat brain was reported by Schanberg, Schildkraut, Breese and Kopin (1968) but these authors were unable to detect the free glycol in this species. Recently Schanberg, Breese, Schildkraut, Gordon and Kopin (1968) have described the determination of MOPEG in brain tissue and cerebrospinal fluid using the method described for urine by Wilk, Gitlow, Clarke and Paley (1967). In this method a trifluoroacetyl derivative of MOPEG is formed and measured by gas chromatography using the electron capture detector.

The formation of acetyl and perfluoroacyl derivatives as the basis of methods for the estimation of catecholamines and their metabolites has been described by Brooks and Horning (1964), Horning, Horning, Vanden Heuvel, Knox, Holmstedt and Brooks (1964) and Wilk, Gitlow, Franklin and Carr (1964). The main problem in applying these methods to biological samples lies in the difficulty of extracting

small quantities of catecholamines and their metabolites from tissues and then achieving the anhydrous conditions required for the introduction of perfluoroacyl and acetyl groupings without excessive losses.

Chattaway (1931) showed that phenolic hydroxyl groups and amino groups could be acetylated very easily in dilute aqueous alkaline solution. This reaction was used by Welsh (1955), Hagopian, Dorfman and Gut (1961) and Laverty and Sharman (1965) as a step in the isolation of catecholamines and their metabolites from aqueous solutions and tissue extracts. The acetylated derivatives are more stable than the parent compounds and thus can be brought more easily to the anhydrous state. Alcoholic hydroxyl groups are not acetylated under these conditions and are left free to form a perfluoroacyl derivative. This paper describes the use of these steps in an approach to the problem of estimating catecholamines and their metabolites in tissues and body fluids by gas chromatography.

## Materials and Methods

The following chemicals and reagents were used:

bis (4-hydroxy-3-methoxyphenylethyleneglycol) piperazine salt (Calbiochem);

3,4-dihydroxyphenylethyleneglycol (Calbiochem);

l-3,4-dihydroxyphenylethanolamine (l-noradrenaline) base (Hoechst A.G.);

d,l-4-hydroxy-3-methoxyphenylethanolamine (D,L-normetanephrine  Calbiochem);

4-hydroxy-3-methoxyphenylethanol (prepared by reduction of 4-hydroxy-3-methoxyphenylacetic acid with lithium aluminum hydride);

γ-benzene hexachloride (Shell);

Heptafluorobutyric anhydride. Prepared from heptafluorobutyric acid (Minnesota Mining and Manufacturing Co. Ltd.) by heating under reflux with phosphorus pentoxide (1 mol. heptafluorobutyric acid : 1.5 moles $P_2O_5$) for 3 hr. Distilled three times B.P. 108°C (uncorrected);

Dichloromethane. May & Baker Ltd. Distilled three times;

Tetrahydrofuran. Hopkin & Williams. Distilled fresh in small quantities as required;

Acetic anhydride. British Drug Houses Ltd. Distilled twice; stored at 4°C;

Methanol. James Burrough Ltd. Purified by distillation from KOH and then redistilled;

Hexane. Hexane fraction, Hopkin & Williams. Distilled twice.

All other chemicals were of analytical reagent quality. Double distilled water was used throughout.

Extraction of tissues

The tissues were removed rapidly after killing the animals and homogenised in ice-cold 0.1N-hydrochloric acid (4 ml. acid for up to 0.5 g tissue). Concentrated perchloric acid (72% w/v), 0.2 ml., was added for each 4 ml. of homogenate and mixed thoroughly. Solid potassium chloride was then added in a slight excess of the amount required for saturation. The homogenate was then

centrifuged with a tip radial acceleration of 147000 $ms^{-2}$ (15000 X *g*) for 5 min at 0°C. The clear supernatant was transferred to a glass stoppered test tube and mixed with 0.3 ml. acetic anhydride. A slight excess of sodium bicarbonate was then added in small portions with mixing, allowing the effervescence to subside between additions. The solution was then extracted twice with 10 ml. portions of dichloromethane which were dried by filtering through anhydrous sodium sulphate. The extract was evaporated to a small volume, under a stream of dry nitrogen, in test tubes standing in holes in an electrically heated aluminium block at 60°C. The concentrated extract was then transferred to a 75 mm X 8 mm borosilicate glass test tube and evaporated to dryness at 60°C under a stream of dry nitrogen.

Formation of heptafluorobutyrate esters

The sample was dissolved in a small volume (20 - 200 µl.) of tetrahydrofuran. One quarter of this volume of heptafluorobutyric anhydride was then added and mixed. The mixture was allowed to stand at room temperature for 5 - 10 min and then evaporated to dryness under a stream of dry nitrogen at 60°C. The gas flow and heating were continued for 15 min after the liquid had disappeared. The formation of the acetyl-heptafluorobutyryl derivatives is thought to proceed as shown in Fig. 1.

Gas chromatography

This was carried out on both Model 400 and Model 402

F & M Scientific Corporation gas chromatographs using in the former a U-shaped column 180 cm X 3 mm i.d. packed with 3.8% SE-30 on silanized Diatoport S (60 - 80 mesh). This column had been conditioned at 300°C for 12 hr and then used at 230°C for several months for the analysis of steroid compounds. The Model 402 was fitted with a new 120 cm X 4 mm i.d. column of 3.8% SE-30 on Diatoport S conditioned by heating at 300°C for 12 hr.

The carrier gas was argon containing 5% methane. A tritium foil electron capture detector was used with both instruments.

The derivatives were dissolved in hexane or in a hexane solution of γ-benzene hexachloride of known concentration for injection into the gas chromatograph. The extracts prepared from tissues were dissolved in volumes of 0.1 - 0.5 ml. of which 0.5 - 2 μl. were injected.

## Results

### The formation of heptafluorobutyryl derivatives of noradrenaline and some catecholamine metabolites

1. 4-Hydroxy-3-methoxyphenylethyleneglycol (MOPEG).

MOPEG was separated from the piperazine in the commercially available salt by dissolving in water, adding hydrochloric acid to bring the pH to 1 - 2 and extracting three times with five volumes of diethyl ether. The ether extracts were dried with anhydrous sodium sulphate and evaporated under a stream of nitrogen, yielding an oily residue. The heptafluorobutyryl derivatives of both MOPEG

and acetyl-MOPEG were prepared from known amounts of MOPEG. Fig. 2 illustrates the separation of these two derivatives on the gas chromatogram and shows that the fully heptafluorobutyrated derivative did not give a response larger than the acetyl-heptafluorobutyryl derivative when these were prepared by the methods described here.

2. Acetyl-heptafluorobutyryl derivatives were prepared from l-noradrenaline, d,l-4-hydroxy-3-methoxyphenylethanolamine (normetanephrine), MOPEG, DOPEG and 4-hydroxy-3-methoxyphenylethanol. The derivatives of noradrenaline, MOPEG and DOPEG were crystallised at -17°C from hexane. The uncorrected melting points of these derivatives were: acetyl-heptafluorobutyryl MOPEG, white crystals 75.5 - 76°C; acetyl-heptafluorobutyryl DOPEG, white crystals 71 - 72°C; acetyl-heptafluorobutyryl noradrenaline, white needles, 93°C.

The retention times of these derivatives relative to γ-benzene hexachloride under differing conditions for chromatography are given in Table 1. Each can be separated from the others on a 3.8% SE-30 column. However, the relative retention time of the noradrenaline derivative is too long at column temperatures below 150°C to allow the measurement of small amounts of noradrenaline and at higher temperatures the response from the derivative of MOPEG tends to overlap with that of the benzene hexachloride, the reference compound. The hexane solutions of the acetyl-heptafluorobutyryl derivatives of MOPEG, DOPEG, nor-

metanephrine and noradrenaline were stable for several months at -17°C if care was taken not to allow water to condense inside the vessels holding the solutions when portions were taken out. The crystalline derivatives were not very stable unless kept under hexane at -17°C.

The quantitative estimation of MOPEG and DOPEG

The principle of the method has been described for the estimation of steroid compounds by Vandenheuvel, Hinderks, Nixon and Layng (1965), Wisniewski and Umbreit (1965) and by Heap, Holzbauer and Newport (1966); it is based on the ratio of the sizes of the responses of two substances, a and b which have different retention times. If the ratio of the sizes of the two responses is proportional to their relative concentrations in a mixture of the two substances and the total amount present in the sample of one of them is known then the total amount of the other substance present in the sample can be calculated from the equation

$$A = \frac{RA}{RB} \times B \times C$$

where A = Total amount of substance a present in the sample,

RA = Size of response to a in the sample,

RB = Size of response to b in the sample,

B = Amount of substance b present,

C = Ratio $\frac{b}{a}$ of responses to equal amounts of a and b.

The last term in the equation is obtained by calculation from the responses observed when samples each containing known amounts of the derivatives both of authentic A

and of authentic B are tested on the gas chromatogram.

In the present experiments benzene hexachloride was selected as the reference compound to be added to the extracts. Fig. 3 shows that the ratios of the heights of the response peaks obtained with the acetyl-heptafluorobutyryl derivatives of MOPEG and DOPEG and γ-benzene hexachloride are suitable for such estimations since the peak height ratios are proportional to the concentration ratios. When peak height ratios were replaced by ratios of areas of the peaks (calculated by triangulation) the variance of the relation between concentration ratio and response ratio was higher because two measurements were required for each area estimation.

Recoveries

Authentic MOPEG was added to tissue homogenates in quantities ranging from 10 - 50 ng to show that any MOPEG present in samples would be detected on the gas chromatogram. In the earlier experiments it was difficult to estimate MOPEG accurately without having the crystalline derivative to obtain accurate standard measurements. In two experiments with the procedure finally adopted in which crystalline standards were used the recovery of MOPEG was 64% and 69% and of DOPEG 67% and 78%.

The presence of MOPEG and DOPEG in the hypothalamus

Hypothalamic tissues of the mouse, rabbit and cat were extracted and the extracts treated to form acetyl-heptafluorobutyryl derivatives. The gas chromatographic record

obtained with an extract of mouse hypothalamus is shown in Fig. 4. This shows that the extract of mouse hypothalamic tissue gives response peaks similar to those of authentic derivatives of MOPEG and DOPEG. The retention times relative to that of the response due to γ-benzene hexachloride are identical with those obtained with the acetyl-heptafluorobutyryl derivatives of MOPEG and DOPEG. The responses correspond, in this experiment, to a concentration in the tissue of MOPEG 0.04 μg/g and DOPEG 0.05 μg/g (uncorrected for recovery). Similar peaks were seen on the recordings obtained with the extracts of rabbit (Sharman, 1969) and cat hypothalamus.

Further evidence for the identity of the substances giving rise to the peaks on the chromatograph record

a) Hydrolysis of the acetyl derivative of MOPEG.

As shown in Fig. 2 the responses of the heptafluorobutyryl derivatives of MOPEG and acetyl-MOPEG are easily distinguished. The acetyl group on the phenolic hydroxyl of MOPEG, introduced during the extraction procedure, can be removed by dissolving in an alkaline aqueous solution. After evaporation to dryness the MOPEG can then be treated to yield the completely heptafluorobutyrylated derivative. A dichloromethane extract containing the acetylated derivatives was prepared from rabbit hypothalamus and from rabbit brain cortex to which MOPEG had been added. Each of these was divided into two equal portions and the four samples evaporated to dryness. To one of each pair of samples was added 0.1 ml. of concentrated ammonia solution and after

5 min these were again evaporated to dryness. All four samples were then treated with heptafluorobutyric anhydride in tetrahydrofuran as described and the final extracts examined by gas chromatography. A peak corresponding with that given by acetyl-heptafluorobutyryl MOPEG was observed with the two extracts that had not been treated with ammonia. The other two extracts showed a response at the retention time corresponding to that of heptafluorobutyryl MOPEG but the size of the response was difficult to estimate as it coincided with the sharp rising phase of another peak on the chromatogram and was observed as a shoulder on this peak.

b) Paper chromatographic separation of the acetyl derivatives before gas chromatography.

A dichloromethane extract containing the acetylated derivatives was prepared from 1.1 g of mouse hypothalamus. One tenth of this extract was taken, evaporated to dryness, treated to form the acetyl-heptafluorobutyryl derivatives and examined on the gas chromatograph. Peaks corresponding with those obtained with acetyl-heptafluorobutyryl derivatives of both MOPEG and DOPEG were obtained similar to those in Fig. 4. The remainder of the methylene dichloride extract was evaporated to a volume of approximately 0.3 ml. This was applied to a 1.5 cm wide lane of Whatman No. 50 filter paper for chromatography which had been washed with 2N-sodium hydroxide and distilled water as described by Sharman (1963). A descending chromatogram was run using a modification of the Bush solvent system C (Bush, 1952). The

solvent system was toluene : methanol : water : ethyl acetate in the proportions 10 : 5 : 5 : 2 and the chromatogram was developed for 2.5 hr at 27°C after equilibrating overnight. After development, the chromatogram was dried and cut up into 1 cm long portions each of which was eluted by intermittent shaking with 1 ml. of methanol which was left in contact with the paper for 1 hr. The pieces of paper were removed, the eluates evaporated to dryness and then treated to form the acetyl-heptafluorobutyryl derivatives. They were then examined by gas chromatography. The result is illustrated in Fig. 5. This shows that those responses on the gas chromatogram at relative retention times similar to those of the acetyl-heptafluorobutyryl derivatives of MOPEG and DOPEG, are only obtained from regions of the chromatograms where acetyl-MOPEG and acetyl-DOPEG are found.

Noradrenaline and normetanephrine

The estimation of these two substances after formation of their acetyl-heptafluorobutyryl esters using gas chromatography is theoretically possible since good responses are obtained when aqueous solutions of either are subjected to the extraction and estimation procedures described for MOPEG and DOPEG.

In practice, the application of the method to measurement of noradrenaline and normetanephrine in tissues has met with technical difficulties. When gas chromatograms of extracts of mouse and rabbit hypothalamus were developed at

140°C and below, a response with a relative retention time similar to that given by the acetyl-heptafluorobutyryl derivative of normetanephrine was observed. However, when the derivative prepared from authentic normetanephrine was added to such extracts, the retention time of the response given by the added normetanephrine derivative could just be distinguished from that of the response seen with the extract alone. Preliminary experiments on eluates from the appropriate regions of the chromatogram used to obtain the results described in Fig. 5 yielded responses, the retention times of which approximated to those of the acetyl-heptafluorobutyryl derivatives of noradrenaline and normetanephrine; but it has not yet been possible to confirm or disprove the identity of these retention times with those of the authentic derivatives. These results indicate that simple extraction procedures are not suitable for the estimation of the two amines by gas chromatography of their acetyl-heptafluorobutyryl derivatives. Moreover the noradrenaline and normetanephrine derivatives are relatively insoluble in hexane which adds to the difficulties in applying the method as given here.

Sensitivity of the method

Fig. 4 shows the responses given by amounts of authentic derivatives corresponding to $50 \times 10^{-12}$ g MOPEG and $100 \times 10^{-12}$ g DOPEG. The responses given by the derivatives of normetanephrine and noradrenaline were, respectively, at least 20 times and 100 times smaller than that given

by the derivative of MOPEG.

Stability of the final extracts

The final hexane extract prepared from a sample of rabbit hypothalamus was tested for its content of acetyl-heptafluorobutyryl MOPEG three times over a period of four days. The extract was kept at -17°C between the tests. On the first day the content of MOPEG was estimated to be 9.6 ng, on the second 9.7 and 9.8 ng and on the fourth day 8.2 ng.

The concentration of MOPEG and DOPEG in the hypothalamus

Table 2 shows the concentration (uncorrected for recovery) of MOPEG and DOPEG estimated to be present in the hypothalamus of several species.

## Discussion

A technique for the estimation of certain catechol-amines which contain a secondary alcoholic hydroxyl group and of some of their metabolites has been developed using a double esterification procedure. The first, an acetylation in aqueous solution, converts the compound into derivatives which are soluble in a water-immiscible organic solvent and the second, the introduction of one or more heptafluoro-butyryl groups, forms derivatives which can be detected by electron capture. The method has proved to be extremely sensitive for the glycol metabolites of noradrenaline. Although it is theoretically possible to use a similar procedure for noradrenaline and adrenaline and their methoxylated amine metabolites, technical problems have

arisen which no doubt can be overcome by further purification of extracts of tissues.

The method for the estimation of the glycol metabolites measures the free glycols present in the tissues and yields values for the concentrations of DOPEG and MOPEG which are not very different in the three species examined. Schanberg, Breese, Schildkraut, Gordon and Kopin (1968) have also used a perchloric acid protein precipitation with results indicating that there is little if any hydrolysis of conjugated MOPEG during this procedure. Preliminary experiments have shown that the simple extraction procedure used here is difficult to apply after treatment of brain extracts with a sulphatase because of the presence of contaminants in the enzyme preparation. Whether the present method is more sensitive than the method of Wilk *et. al.* (1967) will only be decided by a direct comparison but the sensitivity given by these authors (91,000 $mm^2$ for $10^{-9}$ moles) for the response to trifluoro-acetylated MOPEG can be compared with approximately 5,000,000 $mm^2$ for $10^{-9}$ moles, a typical response with the present method.

In addition to confirming the presence of free MOPEG in the central nervous system, the present results show that free DOPEG is also present, a result predicted by experiments on the metabolism of radioactive noradrenaline in the brain.

Table 1. Relative retention times of heptafluorobutyryl esters of noradrenaline and some related compounds.

| | Retention times relative to that of γ-benzene hexachloride | | | | |
|---|---|---|---|---|---|
| Column temperature °C | 127 | | 134 | 140 | 152 |
| Carrier gas flow ml./min | 100 | 100 | 100 | 60 | 100 |
| **Compound** | | | | | |
| 1. Heptafluorobutyryl esters of | | | | | |
| 4-hydroxy-3-methoxyphenylethanol | 0.53 | | | | |
| 4-hydroxy-3-methoxyphenylethyleneglycol (MOPEG) | 0.63 | | | | |
| 2. Acetyl-heptafluorobutyryl esters of | | | | | |
| 4-hydroxy-3-methoxyphenylethanol | 1.05 | | | | |
| 4-hydroxy-3-methoxyphenylethyleneglycol (MOPEG) | 1.33 | 1.46 | 1.31 | 1.40 | 1.15 |
| 3,4-dihydroxyphenylethyleneglycol (DOPEG) | | 2.81 | 2.31 | 2.50 | 1.92 |
| 2-(4-hydroxy-3-methoxyphenyl)ethan-2-olamine (normetanephrine) | | 3.88 | 3.33 | 3.60 | 2.78 |
| 2-(3,4-dihydroxyphenyl)ethan-2-olamine (noradrenaline) | | | | | 4.62 |

Table 2. The concentration of MOPEG and DOPEG in the hypothalamus.

| Species | MOPEG μg/g | | DOPEG μg/g |
|---|---|---|---|
| Rabbit | 0.045 | | Present but not estimated because of lack of standard. |
| | 0.047 | | |
| | 0.159 | | 0.030 |
| | 0.049 | Estimated in duplicate portions of same homogenate. | 0.120 |
| | 0.048 | | 0.126 |
| Mouse | 0.049 | | 0.048 |
| | 0.077 | | 0.054 |
| | 0.040 | | 0.047 |
| | 0.048 | | 0.029 |
| Cat | 0.018 | | 0.022 |
| | 0.017 | | 0.018 |

The observations on mouse tissue were made on pooled samples from six animals.
The observations in rabbit and cat were made on tissues from individual animals.
The duplicate estimations were made on pooled tissues using samples of homogenate equivalent to the tissue from one animal.

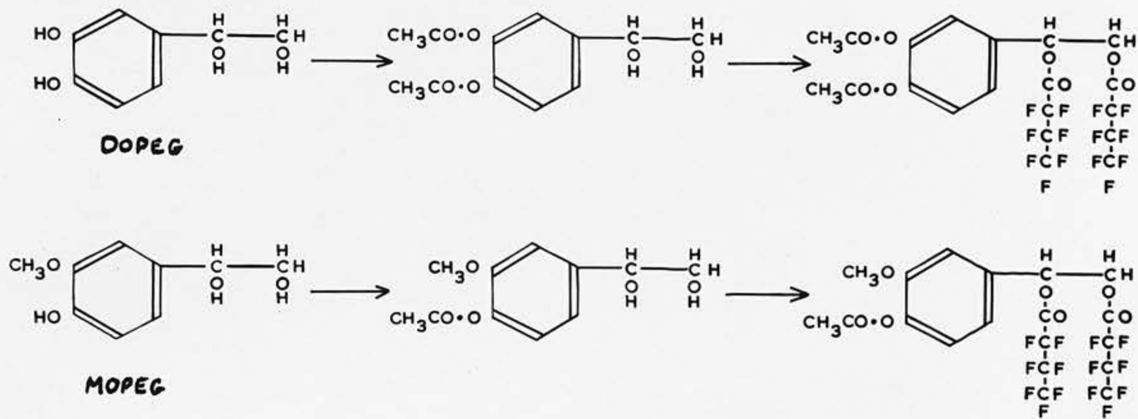


Fig. 1. The formation of acetyl-heptafluorobutyryl derivatives of DOPEG and MOPEG.

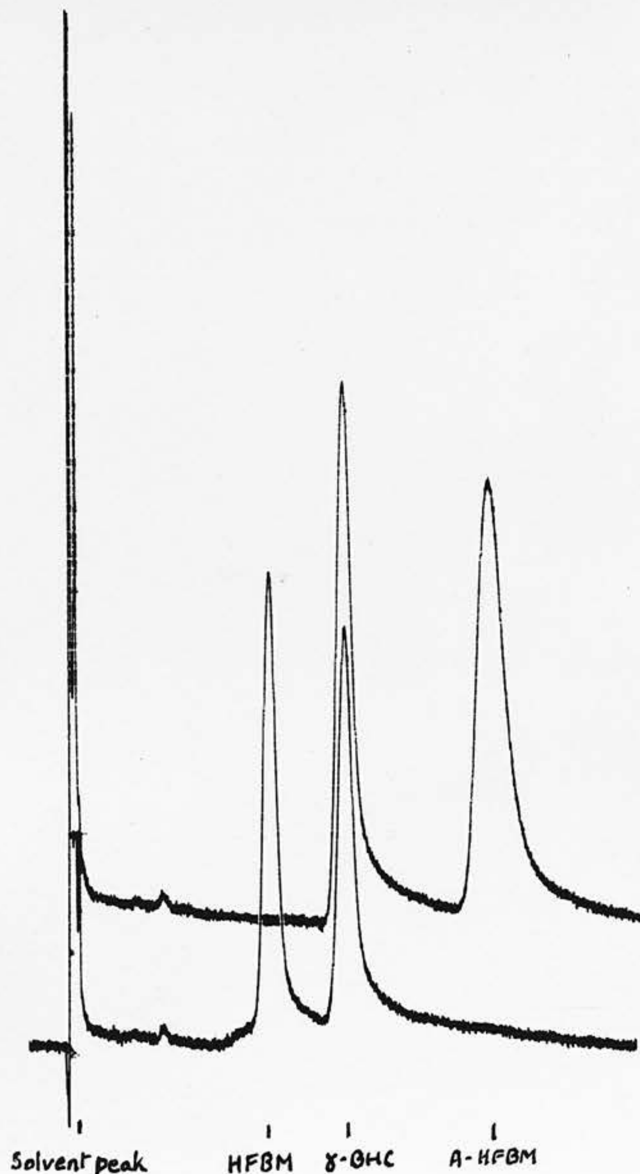


Fig. 2. Gas chromatogram tracings of the fully heptafluorobutyrated derivative of 4-hydroxy-3-methoxyphenylethylene glycol (MOPEG) and the acetyl-heptafluorobutyryl derivative of MOPEG.

The responses were obtained with mixtures containing γ BHC and derivatives of MOPEG in the proportions given in parentheses.

γ-BHC - γ-Benzenehexachloride (4 parts)

HFBM - Heptafluorobutyryl MOPEG (= 7 parts MOPEG)

A-HFBM - Acetylheptafluorobutyryl MOPEG (= 8 parts MOPEG)

Chromatogram conditions:

Column temperature 125°C
Flash heater temperature 160°C
E.C. detector temperature 180°C
Gas flow 100 ml./min at 1.4 kg/cm$^2$ pressure
E.C. detector pulse interval 50 μsec
Column 180 cm x 3 mm 3.8% SE-30

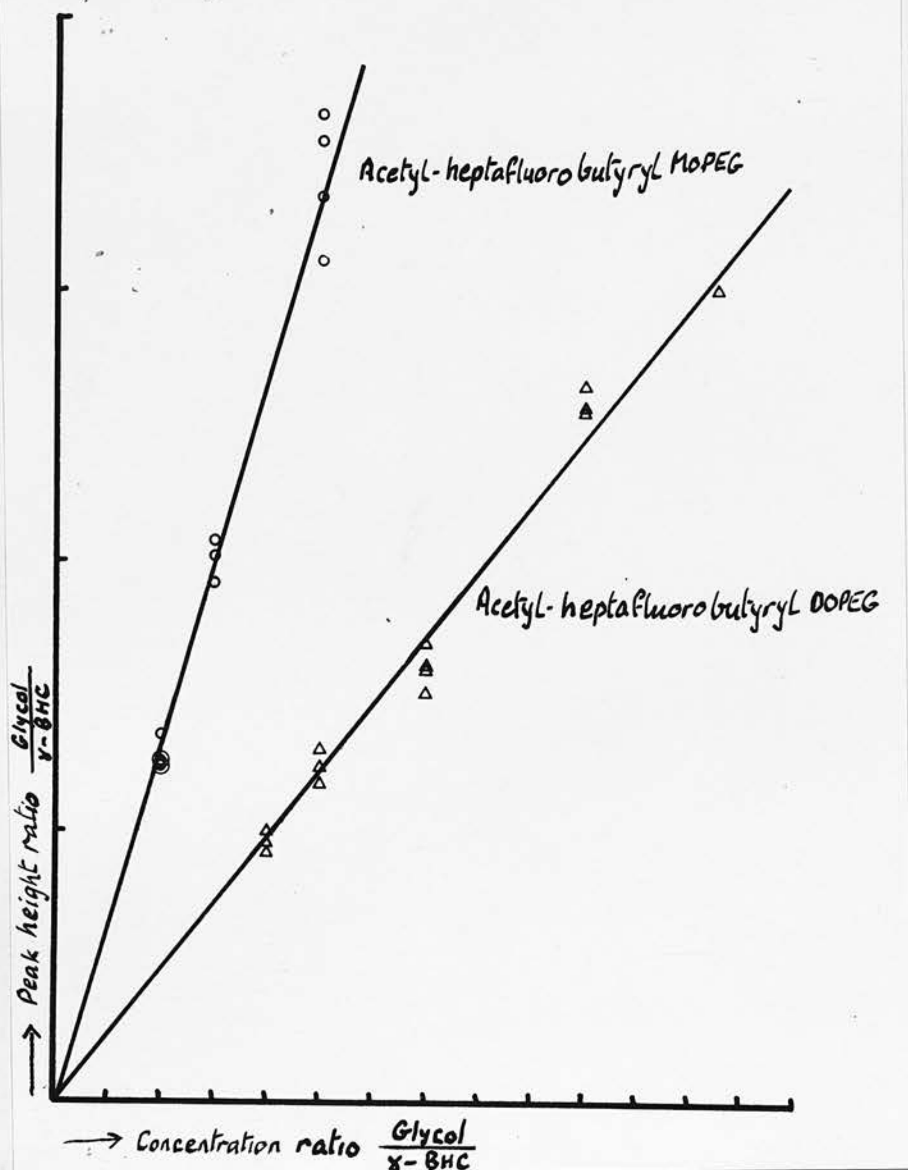


Fig. 3. The ratio of the peak heights of the responses to γ-benzene hexachloride and the acetyl-heptafluorobutyryl derivatives of MOPEG and DOPEG plotted against the ratio of their concentrations in mixtures.

(The concentrations of the derivatives of the glycols are expressed as the concentration of the parent glycol).

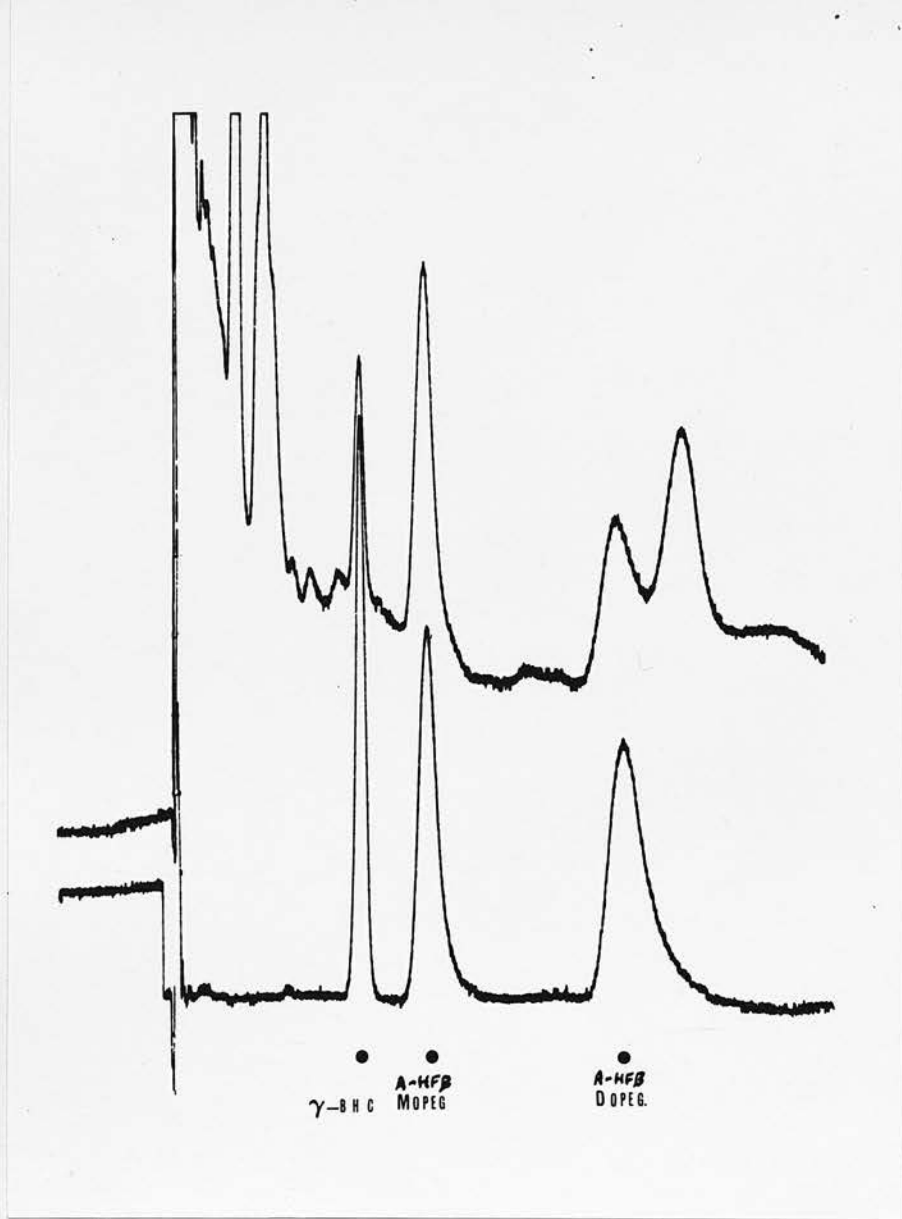


Fig. 4. Gas chromatographic record of an extract of mouse hypothalamus.

Upper tracing: Mouse hypothalamic extract (from 0.103 g tissue) to which 1.25 ng γ-benzene hexachloride had been added.

Lower tracing: Responses to derivatives of authentic MOPEG and DOPEG. Ratio of concentrations BHC : MOPEG : DOPEG = 200 : 250 : 500 ng/ml.
Column temperature 140°C; flash heater temperature 180°C; Detector temperature 170°C; Carrier gas flow 60 ml./min (Argon: 5% methane) (no purge gas) Pulse interval 150 μsec.

Retention times relative to that of γ-benzene hexachloride observed with the extract used for the above record (Mean ± s.e.m. from three observations).

| | | |
|---|---|---|
| Authentic derivatives of | 1) MOPEG | 1.357 ± 0.004 |
| | 2) DOPEG | 2.443 ± 0.002 |
| Peaks observed in extract | 1) MOPEG | 1.367 ± 0.006 |
| | 2) DOPEG | 2.440 ± 0.08 |

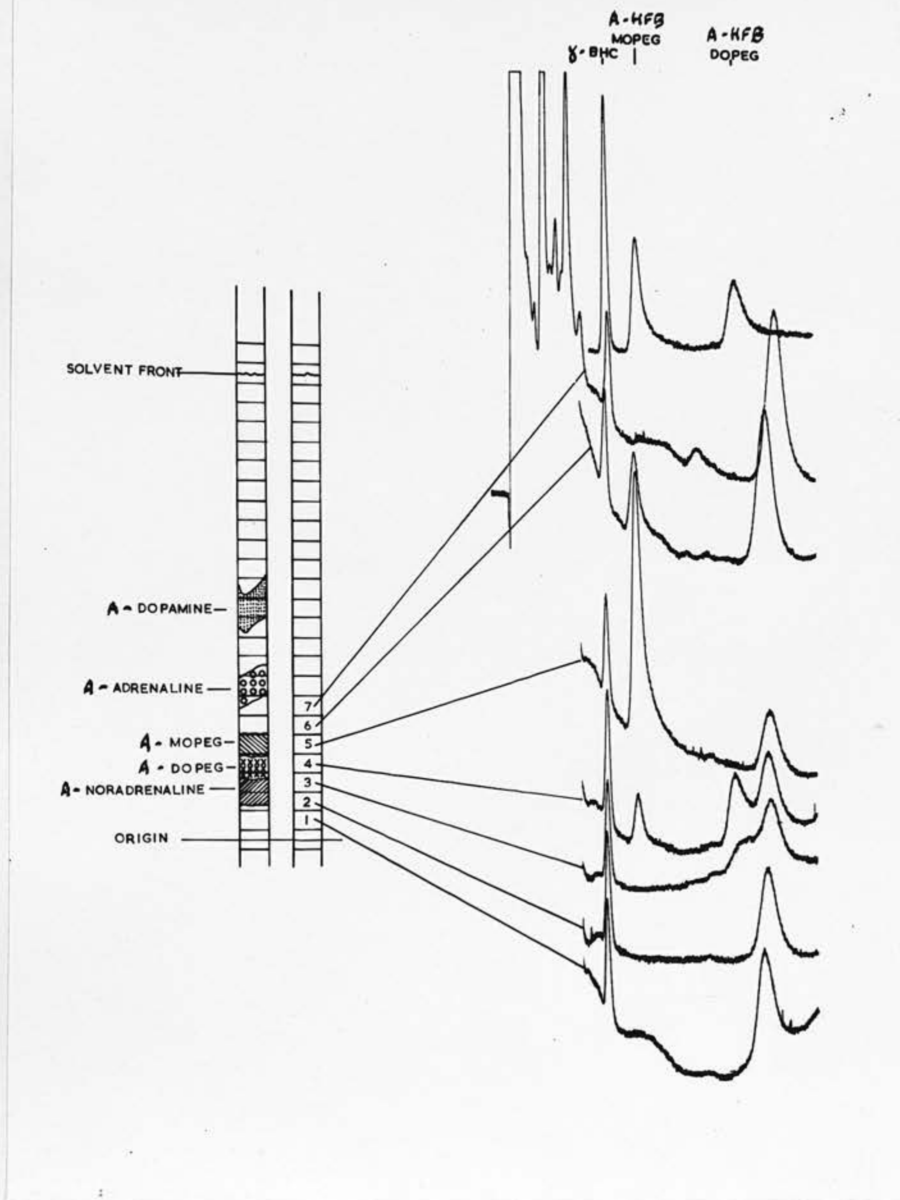


Fig. 5. Gas chromatographic records obtained with eluates from a paper chromatogram of an extract of mouse hypothalamus.

The catecholamines and their metabolites on the tissue were acetylated and separated as indicated on the paper chromatogram by the reference compounds shown in the diagram. The chromatogram was cut into small pieces as indicated by the transverse lines and each piece eluted with methanol. The eluates were evaporated and treated to form hepta-fluorobutyryl derivatives.

No response peaks at retention times relative to that of γ-benzene hexachloride corresponding with those of acetyl-heptafluorobutyryl MOPEG and acetyl-heptafluoro-

butyryl DOPEG were observed with eluates from regions of the chromatogram other than those shown.

The conditions for gas chromatography were similar to those given in Fig. 4.

| | | |
|---|---|---|
| A-Dopamine | - | acetyl dopamine |
| A-Adrenaline | - | acetyl adrenaline |
| A-MOPEG | - | acetyl MOPEG |
| A-DOPEG | - | acetyl DOPEG |
| A-Noradrenaline | - | acetyl noradrenaline |
| γ-BHC | - | γ-benzene hexachloride |
| A-HFB-MOPEG | - | acetyl-heptafluorobutyryl MOPEG |
| A-HFB-DOPEG | - | acetyl-heptafluorobutyryl DOPEG |

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