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

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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).

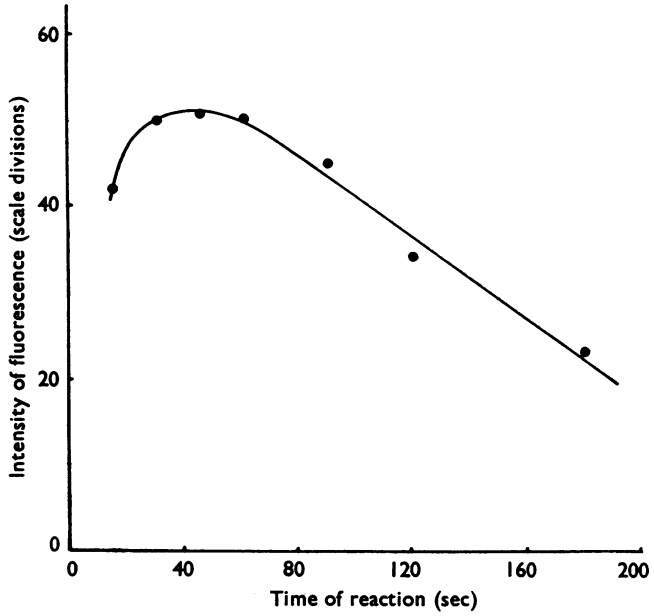

Fig. 1. The effect of the time of reaction (abscissa) with ferric chloride on the development of the fluorescence (ordinate) from homovanillic acid.

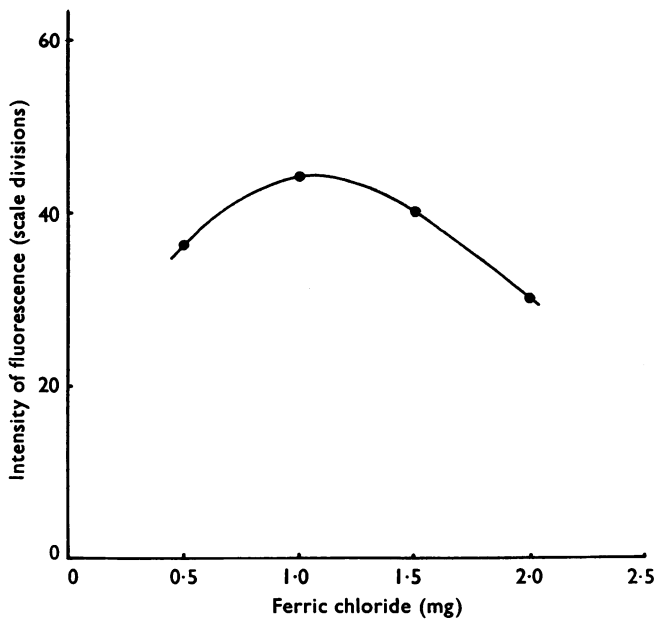

Fig. 2. The effect of the concentration of ferric chloride (abscissa, expressed as mg added) on the development of the fluorescence (ordinate) from homovanillic acid. The ferric chloride was allowed to react for 45 sec.

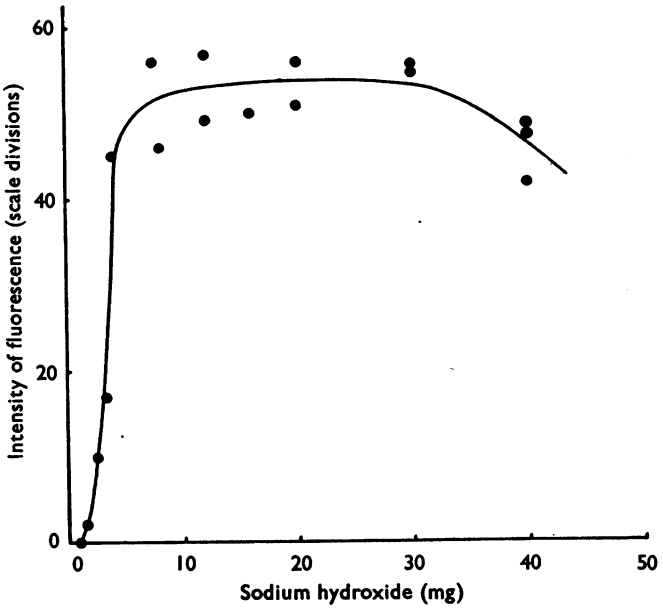

Fig. 3. The effect of the concentration of sodium hydroxide (abscissa, expressed as mg added) on the development of the fluorescence (ordinate) from homovanillic acid.

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 mμ ; fluorescence wavelength 430 mμ) 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

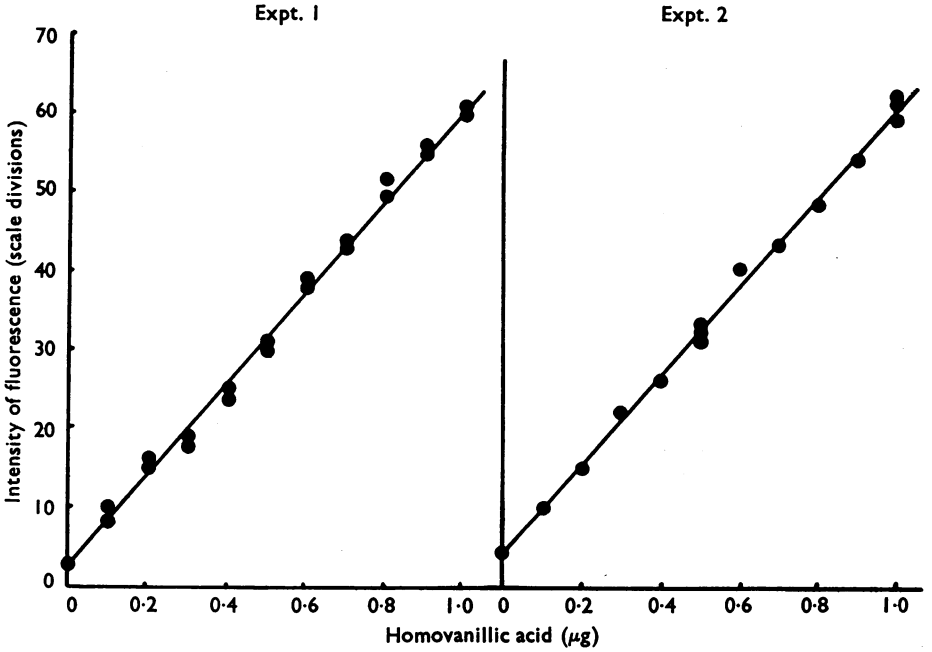

Fig. 4. The relationship between the amount of homovanillic acid (abscissa) and the fluorescence developed (ordinate), for two experiments.

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 mμ, maximum fluorescence wavelength 450 mμ) 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 mμ, fluorescence maximum 450 mμ). 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 μg.

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 *p*-nitroaniline
	A	B	C	D	
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	

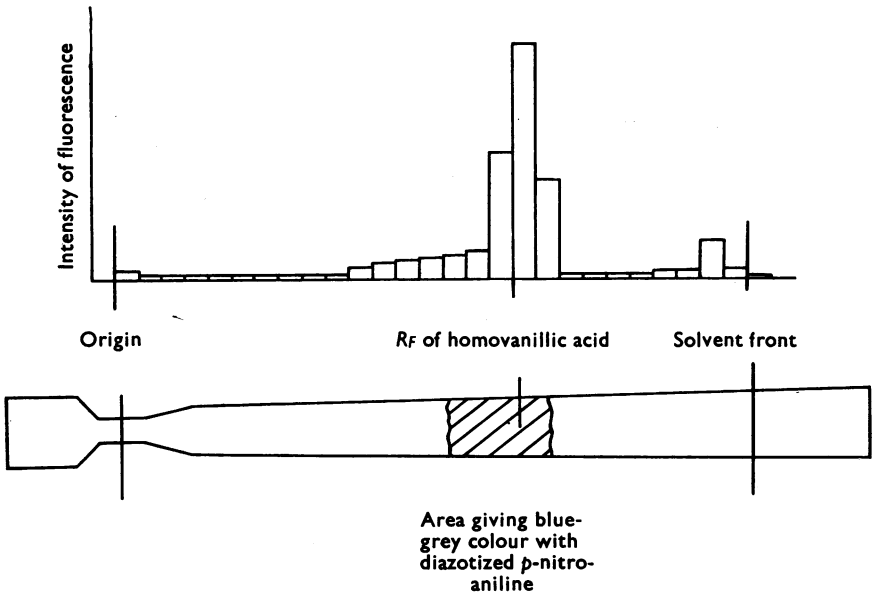

Fig. 5. The localization of apparent homovanillic acid on a chromatogram of an extract of the caudate nucleus of the dog.

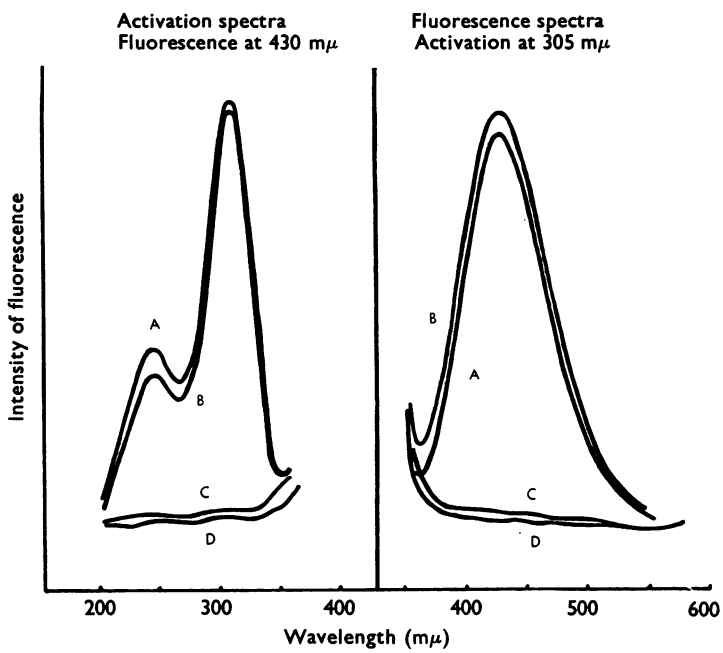

Fig. 6. Activation (left) and fluorescence (right) spectra of the fluorescence developed from the phenolic substance extracted from the caudate nucleus of the dog. A: substance extracted from the caudate nucleus. B: 2 μg of homovanillic acid. C: extract blank. D: reagent blank.

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 (μg/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 μg/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.

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