Br. J. Pharmac. Chemother. (1968), **33**, 441–456.

HOMOVANILLIC ACID, 3,4-DIHYDROXYPHENYLACETIC ACID AND 5-HYDROXYINDOL-3-YLACETIC ACID IN SERIAL SAMPLES OF CEREBROSPINAL FLUID FROM THE LATERAL VENTRICLE OF THE DOG

BY

G. W. ASHCROFT, T. B. B. CRAWFORD, R. C. DOW AND H. C. GULDBERG*

From the M.R.C. Unit for Research in Brain Metabolism, Department of Pharmacology, University of Edinburgh Medical School, Edinburgh

(*Received January* 2, 1968)

Several studies of the concentrations of the acid metabolites of dopamine and 5-hydroxytryptamine in the brain have been reported in recent years. Homovanillic acid (HVA, 4-hydroxy-3-methoxyphenylacetic acid) has been shown to occur in the brain of a number of species and the concentration in various areas of the brain has been measured. (Sharman, 1963 ; Andén, Roos & Werdinius, 1963a ; Gottfries, Rosengren & Rosengren, 1965 ; Juorio, Sharman & Trajkov, 1966). The basal ganglia contain particularly high concentrations of HVA in the dog, and these areas are also rich in dopamine (Laverty & Sharman, 1965). Another acid metabolite of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), has also been demonstrated in several species in areas of the brain, such as the corpus striatum, which are rich in this amine (Rosengren, 1960). In the corpus striatum of the rabbit, DOPAC occurred in a concentration about one tenth of that of HVA (Andén, Roos & Werdinius, 1964) and a similar relationship has been found in the caudate nucleus of the dog (Guldberg, unpublished data). 5-Hydroxyindol-3-ylacetic acid (5-HIAA) has been shown to occur in the brain of the dog and other species. In the dog, areas with high amounts of 5-hydroxytryptamine also have high concentrations of 5-HIAA (Eccleston, Ashcroft, Moir, Parker-Rhodes, Lutz & O'Mahoney, personal communication).

With regard to the presence of these acids in cerebrospinal fluid (C.S.F.), the data in the literature are less extensive. HVA has been demonstrated in human lumbar C.S.F. (Andén, Roos & Werdinius, 1963b ; Bernheimer, Birkmayer & Hornykiewicz, 1966) and in dog C.S.F. (Guldberg, Ashcroft & Crawford, 1966). There is no report of the presence of DOPAC in C.S.F. Ashcroft & Sharman (1960) found 5-hydroxyindolyl compounds in human lumbar C.S.F. and subsequently (Ashcroft & Sharman, 1962) in dog C.S.F. obtained from punctures in the region of the cisterna magna.

In the present paper a technically simple procedure adapted from existing methods to permit the estimation of the three acids, HVA, DOPAC and 5-HIAA in a single small

* Present address: Department of Pharmacology, University of Bergen, Bergen, Norway.

sample of lateral ventricular C.S.F. from the dog is described. Application of the technique to the determinations of the concentrations of the acids in dog C.S.F. is reported together with a study of the effects of the removal of C.S.F. samples, repeated at relatively short intervals, on the acid concentrations in the fluid. By repeated sampling and determination of the acid concentrations in each sample, it was hoped to obtain a dynamic picture, over a period of time, of drug-induced changes in the metabolism of the parent amines in the brain without recourse to multiple acute experiments involving brain analyses. Such application of the technique is illustrated in the following paper (Guldberg & Yates, 1968) in which are described studies of the C.S.F. acid concentrations at intervals following the administration to dogs of various drugs already known to affect the metabolism of dopamine and 5-hydroxytryptamine in the brain.

Qualitative evidence, based on thin-layer chromatography, for the presence of HVA, DOPAC and 5-HIAA in the lateral ventricular C.S.F. of the dog is presented. The possible occurrence of other metabolites from dopamine, noradrenaline, tyramine and 5-hydroxytryptamine was also investigated, with negative results.

METHODS

Sampling of C.S.F. from the lateral ventricle of the dog

Beagle dogs, 2–3 years old, of both sexes, and weighing about 10 kg were used. To facilitate the withdrawal of C.S.F., guide tubes for the sampling needles were implanted in the skull at a preliminary operation. The technique of operation was that described by Manuilov (1958), with some small modifications. The dogs were anaesthetized with sodium pentobarbitone (Veterinary Nembutal, Abbott) intravenously and intubated with an endotracheal tube to prevent obstruction of the air-way by external compression during the operation. Strict aseptic conditions were adhered to throughout the operation. A longitudinal incision, 3–4 cm in length, was made about 2.5 cm from the mid-line. The lateral position of the incision was necessary to avoid the scar being over the guide tube. The vault of the skull was then exposed ; the temporal muscle was divided along the line of its attachment to the temporal bone and was dissected from the bone for a distance of 0.5 cm laterally. Instead of using the fronto-temporal suture as a landmark for positioning the burr-hole in the skull (Manuilov, 1958) it was found more convenient to obtain the location by measuring 3.8 cm forward in the midline from the caudal surface of the external occipital protuberance and laterally 0.5 cm from the midline. The burr-hole was drilled perpendicular to the top of the skull using a hand-drill No. 30 and it should then be situated over the posterior part of the lateral ventricle where the inferior horn gives good depth within the compartment (Fig. 1). The opening in the skull was tapped, using a 3BA tap, the pointed end of the tap just piercing the dura mater. The stainless steel guide tube (Fig. 2) was inserted and the lower part of the head of the guide tube screwed into the trephine opening using a box spanner specially made to fit the head of the guide tube.

When the guide tube had been fixed in position, a test puncture was made. A sterile stainless steel hypodermic needle (Fig. 3) with a stilette in position was introduced through the guide tube. When the stilette was removed, C.S.F. flowed freely if the tip of needle was properly situated in the ventricle. Routinely, a guide tube to the other lateral ventricle was also inserted and tested similarly for its location. The needles were removed from the guide tubes and the aponeurosis and the skin were sutured in layers. The skin sutures were removed under thiopentone anaesthesia 5–7 days after the operation. At the same time tests were carried out to ensure that C.S.F. could be obtained on passage of a suitable sterile hypodermic needle with a stilette in position through each of the guide tubes. On withdrawal of the stilette, C.S.F. should flow freely from the hub of the needle and 0.5–1.0 ml. should be readily collected. The sampling needles were of standard length but not infrequently it was found that when the needle was inserted C.S.F. would not flow until the needle had been withdrawn for a few mm. The needle was then shortened accordingly to the length appropriate to the particular dog. The side having the best flow was usually chosen for

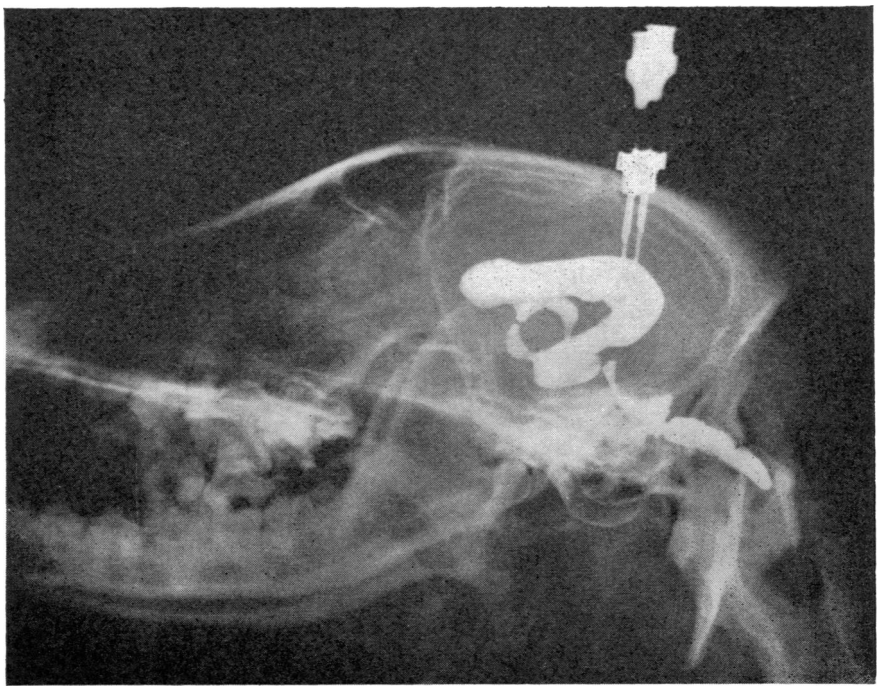
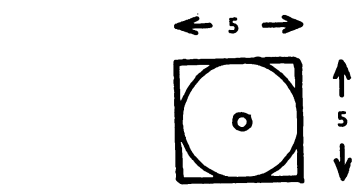


Fig. 1. X-ray photographs of the ventricular system of the dog's brain. The intraventricular system was visualized after an injection of "Myodil" through one of the needles introduced in the indwelling guide tube. Note that the guide tubes, one for each of the lateral ventricles, are situated over the inferior horns of the lateral ventricles.



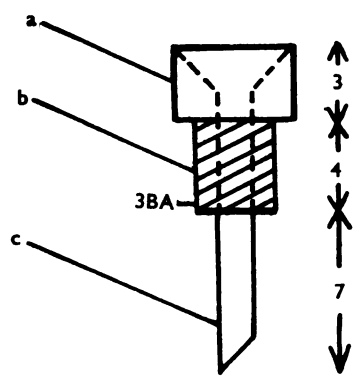


Fig. 2. Guide tube for the lateral ventricle in the dog brain (sectional view). a, Part situated subcutaneously ; b, part screwed into skull ; c, part inserted in the brain directed towards lateral ventricle. 17 gauge tube, 1 mm. bore. All measurements are in mm.

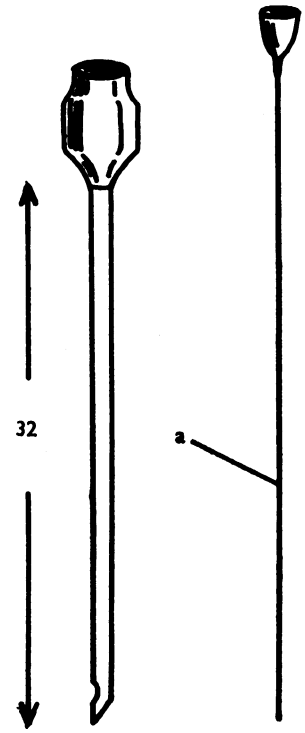


Fig. 3. Luer fitting needle, 21 gauge, with side hole. a, Stainless steel stilette, 25 gauge. All measurements are in mm.

the experiments, but on occasions it was necessary to sample from the other ventricle when it proved difficult to obtain the free flowing C.S.F. without blood contamination. It was found that the risk of traumatic bleeding was higher when the guide tube was inserted too far anteriorly, presumably because the tip of the needle was then in a shallower part of the ventricle and thus more likely to traumatize the choroid plexus. Dogs which were found unsuitable for C.S.F. sampling, because of either poor flow from both lateral ventricles or frequent bleeding into the C.S.F., were operated on again, to change the angle of the guide tube slightly or to make new burr-holes for relocation of the guide tubes.

At least 2 weeks were allowed to pass between the initial operation and experiments involving the sampling of C.S.F. To sample the C.S.F., the sterile stainless steel hypodermic needle, with the stilette in position, was introduced through the guide tube. To obtain a single sample of fluid, the stilette was removed, a sterile 1 ml. disposable syringe fitted to the hub of the needle and the C.S.F., usually 0.5 ml., withdrawn slowly without the application of any appreciable negative pressure. The syringe and needle were then removed. For repeated sampling at intervals of 1 hr or less, the needle was left *in situ* for the duration of the experiment. Into the hub of the needle was inserted the nozzle, cut off with a short length of the barrel still attached, from a 1 ml. "Tuberculin" disposable syringe. The short length of barrel was of sufficient depth to accommodate a rubber plug—the rubber plunger from the same syringe and disconnected from the handle served this purpose (Fig. 4). These fittings were sterilized before use. Each sample of C.S.F. was withdrawn into a sterile disposable 1 ml. syringe fitted with a 26 gauge needle which pierced the rubber plug.

The sample of C.S.F. was transferred to a glass test-tube fitted with a glass stopper and stored at below 0° C until the analysis could be performed. If the C.S.F. contained a small amount of blood it was centrifuged immediately and if there was no sign of haemolysis the colourless supernatant fluid was removed and stored for analysis. If the sample showed haemolysis after centrifugation or was heavily blood-stained on withdrawal the sample was discarded, for experience showed that such contamination interfered with the subsequent assays.

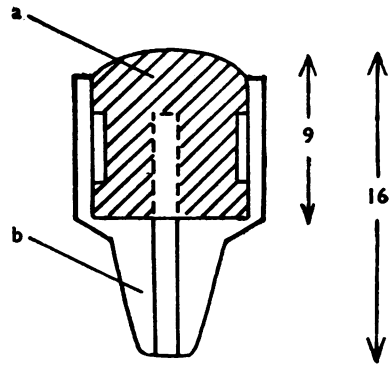


Fig. 4. Sampling diaphragm. a, Rubber diaphragm; b, Luer fitting nozzle and attached body cut from 1 ml. plastic syringe. All measurements are in mm.

Most of the experiments with repeated sampling at fixed time intervals were carried out with the animal under light anaesthesia induced and maintained by sodium thiopentone administered intravenously. The depth of anaesthesia was judged from clinical signs related to effects on muscle tone, reflexes and respiration, etc. In a few instances the C.S.F. samples were obtained from the conscious animal. An interval of at least a week was allowed to elapse between experiments on any one dog. The dogs have tolerated these experiments very well, some having been used for weekly experiments for more than a year.

Analytical methods

Deionized distilled water was used throughout. Reagents were of Analar or, in the case of concentrated hydrochloric acid, Micro-analytical Reagent grade (British Drug Houses), with the exception of ethylenediamine, which was of Laboratory Reagent (B.D.H.) grade and distilled less than 48 hr before use.

Extractions and the development of fluorophors from the acids were carried out in glass tubes fitted with glass stoppers.

Solvent extraction procedure for the phenolic acids in C.S.F.

The frozen sample of ventricular C.S.F. was thawed and a known volume, usually 0.5 ml., was diluted to 2.5 ml. with water. After acidification with 0.02 ml. of concentrated HCl, the solution was saturated with sodium chloride, about 1 g being required. The mixture was extracted twice with 10 ml. of ethyl acetate, on each occasion being shaken for 5 min and then centrifuged at 2,500 rev/min for 5 min. The phenolic acids in the combined ethyl acetate extracts were then transferred to aqueous solution by shaking with 3.2 ml. of 0.1M Tris-hydroxymethylaminomethane buffer (Tris buffer), pH 8.6, for 5 min followed by centrifugation at 2,500 rev/min for 5 min. The aqueous phase was used for the fluorimetric estimation of each acid.

In order to measure the recovery of the acids through the extraction procedure, 200 mμg each of HVA, DOPAC and 5-HIAA was added to selected C.S.F. samples. This was done with each batch of C.S.F. samples analysed.

Spectrophotofluorimetric estimation of homovanillic acid, 3:4-dihydroxyphenylacetic acid and 5-hydroxyindol-3-ylacetic acid

An Aminco-Bowman spectrophotofluorimeter was used to measure the fluorophors. Activation and fluorescence wavelengths quoted are uncorrected values.

A 1.0 ml. portion of the aqueous phase obtained from the extraction procedure described above was used for the estimation of its content of one of the acids.

Homovanillic acid was estimated by the method described by Andén *et al.* (1963a). The acid is converted to a fluorophor by treatment with potassium ferricyanide in ammoniacal solution, the excess oxidizing agent being removed after a set time by the addition of cysteine. To the 1.0 ml.

portion of C.S.F. extract was added 1.0 ml. of 5N ammonia solution and the oxidation was performed by the addition of 0.2 ml. of 0.01% (w/v) potassium ferricyanide. After exactly 4 min, 0.2 ml. of 0.1% (w/v) cysteine was added. The samples were read within 15 min for their fluorescence intensity at 325 mμ/430 mμ (activation/fluorescence wavelengths).

3:4-Dihydroxyphenylacetic acid was estimated by the method of Rosengren (1960). To the 1.0 ml. portion of C.S.F. extract was added 0.5 ml. of a freshly prepared mixture of redistilled ethylenediamine and 4M ammonium chloride (1:1.3 v/v) and the resulting solution heated in a closed tube in a water bath at 50° C for 20 min with constant shaking and with the exclusion of light. The solution was cooled to room temperature and its relative fluorescence measured at 425 mμ/530 mμ (activation/fluorescence wavelengths) in comparison with those of standards processed concurrently.

5-Hydroxyindol-3-ylacetic acid was estimated by the method of Ashcroft & Sharman (1962). To the 1.0 ml. portion of C.S.F. extract was added 0.5 ml. concentrated HCl containing ascorbic acid (0.5 mg/ml.). The fluorescence intensity at 310 mμ/550 mμ (activation/fluorescence wavelengths) was measured in comparison with standard solutions of the acid.

Estimation of the "blank" values for the analyses. With each batch of analyses "blank" extracts were prepared by substituting water for the C.S.F. samples in the extraction procedure and the appropriate "extraction blank" for each acid was determined in the extract so obtained.

"Tissue blank" estimates for C.S.F. extracts were feasible only in the case of HVA by the method described by Andén *et al.* (1963a) in which the cysteine is added to the C.S.F. extract before the ferricyanide, thus preventing fluorophor formation from the acid.

"Reagent blanks" applicable to the measurements of standard solutions of the acids contained only these reagents necessary for fluorophor formation.

Qualitative chromatography of extracts of C.S.F.

Preparation of C.S.F. extracts for thin layer chromatography. A sample (1 ml.) of ventricular C.S.F. was taken from each of two dogs and the sampling repeated at hourly intervals to give five samples from each dog. The samples were pooled and one half of the pooled C.S.F. was processed as such while the other half was processed after the addition of 5 μg of each of the following phenolic acids: HVA, DOPAC, 5-HIAA, *p*-hydroxyphenylacetic acid (pOHPAC), 4-hydroxy-3-methoxymandelic acid (vanillinmandelic acid, VMA), 3,4-dihydroxymandelic acid (DOMAC) and *p*-hydroxymandelic acid (pOHMAC). The latter mixture constituted the "marker sample."

Each pooled C.S.F. sample was acidified to pH 2 with HCl, saturated with sodium chloride and extracted twice with 2 vol. of ethyl acetate, centrifuging between each extraction as described previously. The acids in the combined ethyl acetate extracts were returned to aqueous solution by shaking with 5 ml. Tris buffer, 0.1M and pH 8.6, for 5 min. After centrifugation, the ethyl acetate layer was removed and in certain instances kept for examination for the presence of alcohol metabolites of dopamine and 5-hydroxytryptamine such as 4-hydroxy-3-methoxyphenylethanol and 5-hydroxytryptophol respectively. The aqueous phase was acidified with HCl to pH 2, saturated with sodium chloride and extracted twice with 10 ml. portions of ethyl acetate. To the combined ethyl acetate extracts anhydrous sodium sulphate was added to remove any water present. The supernatant fluid was decanted and evaporated to dryness in a stream of nitrogen at room temperature.

Thin-layer chromatography. The residue of the ethyl acetate extracts was dissolved in 0.1 ml. 80% (v/v) methanol and applied to silica gel H (E. Merck A.G.) thin-layer plates, evaporation of the solvent being assisted by a stream of nitrogen. Pure solution markers of the authentic acids were also applied to the plates.

The residue from the ethyl acetate extract which would contain non-acid phenols was transferred to the silica layer in a similar manner. Pure solutions of 4-hydroxy-3-methoxyphenylethanol and 5-hydroxytryptophol as separate spots, were applied in parallel.

The chromatograms were developed in the lower (organic) phase of a mixture of chloroform:acetic acid:water (2:2:1, by vol.) after equilibration for 1 hr. To effect adequate separation of the phenolic

acids, the chromatograms were developed three times in the same solvent in the same direction with intermediate drying and allowing time for equilibration within the chromatography tank before each run ("multiple thin layer chromatography," Randerath, 1963).

Some C.S.F. extracts were subjected to two-dimensional thin-layer chromatography using the above chloroform:acetic acid:water mixture as the first solvent system followed by propan-2-ol: ammonia (S.G. 0.88):water (8:1:1 by vol.) as the second solvent.

Identification of the phenolic acids on thin-layer chromatograms

The identification of the acids in the C.S.F. extracts was dependent on their positions on the chromatograms and on their reactions to the various test reagents referred to below, the comparisons being made with the authentic substances run in parallel on the chromatograms.

Before applying the test reagents, the solvent was allowed to evaporate off in air. It was necessary, if the solvent contained acetic acid, to remove this acid thoroughly. This was achieved by leaving the plate for at least an hour in a partially closed tank into which was passed a rapid stream of nitrogen. The following reagents were used:

1. Diazotized p-nitroaniline reagent. The plate was sprayed with a freshly prepared mixture of 20 ml. saturated solution of *p*-nitroaniline in 0.1N-HCl and 1.2 ml. of 5% (w/v) sodium nitrite. After drying, the plate was sprayed with 5N sodium hydroxide in 50% (v/v) methanol. This reagent was used for the detection of phenolic substances.

2. Ferricyanide reagent. Spraying the developed chromatogram with 0.1% potassium ferricyanide in 5N ammonium hydroxide yielded a bright blue fluorescence in ultraviolet light with small amounts of HVA.

3. Ethylenediamine reagent. The plate was sprayed with a mixture of ethylenediamine and 4M ammonium chloride (1:1.3 v/v) diluted to 5 vol. with water. The silica gel layer was then covered with a clean glass plate and heated at 80° C for 30 min. This reagent was used essentially for the detection of substances containing catechol groups, the chromatogram being examined under ultraviolet light. Fluorescent substances, however, were also produced from 5-HIAA and from VMA under these conditions.

4. 2:6-dichloro-benzoquinone-4-chloroimide reagent. The plate was sprayed with a 0.1% solution of 2:6-dichloro-benzoquinone-4-chloroimide in absolute ethanol, followed after drying by 5% (w/v) potassium carbonate. This reagent was principally used for the detection of small amounts of the mandelic acids.

5. Ehrlich's reagent. A freshly prepared solution of 10% (w/v) *p*-dimethylaminobenzaldehyde in a mixture of concentrated HCl and acetone (1:4 v/v) was used for the detection of indoles.

6. Hydrochloric acid reagent. The developed chromatogram was sprayed with a solution of 2 ml. concentrated HCl diluted to 25 ml. with acetone. Examination under ultraviolet light revealed 5-hydroxyindolic compounds as pink fluorescing areas.

RESULTS

Spectrophotofluorimetric methods

Linearity and threshold sensitivity of the methods. The relationship between relative fluorescence intensity and quantity of acid was examined with pure solutions of the acids over the range 50–1,000 mμg for HVA, 50–500 mμg for DOPAC and 50–400 mμg for 5-HIAA. In each case the relationship was linear.

"Reagent blank" values were equivalent to about 25 mμg for HVA, 35 mμg for DOPAC and 20 mμg for 5-HIAA.

Sample estimates were usually of the order of 300 mμg for HVA, 50 mμg for 5-HIAA and 50 mμg for DOPAC, this last being at the lowest limit of sensitivity of the method.

Blanks. " Extraction blanks " for the estimations of all three acids gave average readings of 1.4–1.6 times the " reagent blank."

The " tissue blank " for the HVA method was found to be not significantly different from the " extraction blank " when using 0.5 ml. C.S.F. It has been assumed that this is true also for the DOPAC and 5-HIAA estimations, and the fluorimetric readings for each acid in a C.S.F. extract were corrected for a " tissue blank " equal to 1.5 times the " reagent blank." This assumption may not be justified and hence the concentrations of the acids as quoted may be in error to some degree. For most of our experiments, however, we were concerned with the relative concentrations of each acid in serial samples of C.S.F. and in these circumstances inaccurate correction for the " tissue blank " in the series of estimates would be of no consequence provided that the blank did not alter from sample to sample. That the last statement is probably true is borne out by the constancy of the estimates from serial samples withdrawn at intervals of 1 hr.

Specificity of methods of analysis. Samples (1 μg) of DOPAC, DOMAC, pOHPAC, pOHMAC, VMA and 5-HIAA were tested for fluorophor production under the conditions used for HVA. None gave a fluorescence with the characteristics of the homovanillic acid fluorescence and none contributed to the blank reading. 4-Hydroxy-3-methoxyphenylethanol has the same fluorescent characteristics as HVA following ferricyanide oxidation. By the method of extraction used, however, the alcohol would not be present in the final extract in significant concentration.

Similarly, 1 μg amounts of HVA, pOHPAC, VMA, DOMAC and 5-HIAA were tested in the ethylenediamine condensation reaction used for DOPAC. It was ascertained that none would interfere with the estimation of the fluorophor of DOPAC except the catechol, DOMAC. The fluorophor obtained from C.S.F. extracts, having the characteristics of that of a catechol acid, was therefore tested for its partition coefficient between Tris buffer, 0.1M and pH 8.6, and iso-butanol (Weil-Malherbe, 1959). This was found to be approximately 1, the same as that from authentic DOPAC, while the partition coefficient for the fluorophor from DOMAC was 0.1. It may thus be concluded that the DOMAC could not account for an appreciable fraction of the catechol acids in the C.S.F. extract.

3,4-dihydroxyphenylethanol gave the same fluorescent characteristics as the catechol acids using the ethylenediamine method, but should not be present in the final extract.

HVA and DOPAC when present in amounts of 1 μg did not interfere in the fluorimetry of 5-HIAA.

Recovery experiments. In ten experiments the mean percentage recovery $\pm$S.D. of 200 mμg amounts of each acid added to C.S.F. samples was as follows: HVA, 95 ± 3.1; DOPAC, 81.4 ± 5.8; 5-HIAA, 84.4 ± 1.5.

The recovery results for each acid were consistent, the standard deviations being less than 7.5% of the mean percentage recovery. These data were obtained by one operator and the variability in different hands might well be larger. The values for the acid concentrations in the C.S.F. from normal dogs quoted in Table 2 were obtained by the same operator and, consequently, corrections have been made for the recoveries, using the mean recovery figures.

Identification of HVA, DOPAC and 5-HIAA in C.S.F.

Using thin-layer chromatography on silica gel H, it was found that 0.5–1.0 μg of the acids could be detected in the developed chromatogram. The solvent system, the organic phase of a chloroform : acetic acid : water (2 : 2 : 1 by vol.) mixture, gave satisfactory separation of several of the phenolic acids. The R_F values of the acids are recorded in Table 1, column 1. By applying the " multiple " chromatographic technique, with three developments with the solvent, DOMAC moved from the origin and better separation of the mandelic acids was achieved (Table 1, column 2).

TABLE 1

THIN-LAYER CHROMATOGRAPHY OF SOME PHENOLIC ACIDS ON SILICA GEL H USING ORGANIC PHASE OF A CHLOROFORM: ACETIC ACID: WATER (2 : 2 : 1, BY VOL.) MIXTURE

Solvent front at 14 cm from origin with each development with the solvent.

Phenolic acid	Single development R_F	Distance (cm) from origin after three repeated developments with solvent (" multiple chromatography ")
Homovanillic acid	0·60	12·3
3,4-Dihydroxyphenylacetic acid	0·23	7·5
p-Hydroxyphenylacetic acid	0·43	11·2
Vanillinmandelic acid	0·14	5·0
3,4-Dihydroxymandelic acid	at origin	1·4
p-Hydroxymandelic acid	0·08	3·3
5-Hydroxyindol-3-ylacetic acid	0·32	9·2

Chromatograms of C.S.F. extracts showed the following characteristics. There was material present corresponding in position to HVA and reacting with the test reagents as follows. With alkaline diazotized *p*-nitroaniline it gave a greyish red colouration, with the ferricyanide reagent and with the ethylenediamine reagent it fluoresced bright blue and pale blue respectively in ultraviolet light. These reactions were similar to those of authentic homovanillic acid. Using the ethylenediamine reagent, we obtained evidence of the presence of DOPAC in the extract from ventricular C.S.F. The acid could just be detected in C.S.F. extracts using the diazotized *p*-nitroaniline reagent but the colour reaction was inconclusive.

In the developed chromatograms of C.S.F. extracts material in the position of the marker 5-HIAA gave reactions similar to this acid. Both showed a marked pink fluorescence in ultraviolet light after treatment with the hydrochloric acid reagent, a blue colour with the Ehrlich reagent and a green fluorescence in ultraviolet light after reaction with the ethylenediamine reagent. The amount of 5-HIAA present in the C.S.F. extract chromatogram was insufficient to yield a characteristic colour reaction with diazotized *p*-nitroaniline.

Two-dimensional chromatography of a C.S.F. extract using chloroform : acetic acid : water (2 : 2 : 1 by vol., organic phase) as solvent 1 and propan-2-ol : ammonia sp. gr. 0.880 : water (8 : 1 : 1 by vol.) as solvent 2, showed the presence of material in the positions of HVA, DOPAC and 5-HIAA, in both solvent systems indicating that the substance detected in the C.S.F. extracts at the position of each of these acids in the one-dimensional chromatograms was probably not a mixture.

We were unable to identify any other phenolic acids in the C.S.F. extracts. With the diazotized *p*-nitroaniline reagent there were on several occasions two faint spots, with

uncharacteristic colours, close to the positions of the marker areas of VMA and DOMAC respectively. Neither, however, gave the characteristic blue colour which is formed from VMA using the dichloro-*p*-benzoquinone chloroimide reagent or the yellow fluorescence in ultraviolet light given by DOMAC on treatment with the ethylenediamine reagent.

In the extract fractions from C.S.F. which might contain the larger proportions of any phenolic alcohols we were unable to find evidence for the presence of 4-hydroxy-3-methoxyphenylethanol or 5-hydroxytryptophol. The procedure for the thin-layer chromatography of the neutral substances was the same as for the phenolic acids using chloroform : acetic acid : water (2 : 2 : 1 by vol.), with the "multiple" development technique. 4-Hydroxy-3-methoxyphenylethanol was located with the *p*-nitroaniline reagent and the 5-hydroxytryptophol using Ehrlich's reagent, as described previously.

Concentrations of HVA, DOPAC and 5-HIAA in the ventricular C.S.F. of dogs

In Table 2 the concentrations of HVA, DOPAC and 5-HIAA in the ventricular C.S.F. of eight dogs are shown. The acid concentrations for each dog are the means of estimates from single samples withdrawn from the dog at intervals of at least 8 days. The grand

TABLE 2

CONCENTRATION OF HOMOVANILLIC ACID (HVA), 3,4-DIHYDROXYPHENYLACETIC ACID (DOPAC) AND 5-HYDROXYINDOL-3-YLACETIC ACID (5-HAIA) IN LATERAL VENTRICULAR C.S.F. OF THE DOG

The value of each acid for each dog is the mean ± standard deviation of estimates from single samples withdrawn from the dog on various days (No. of estimates). The interval between withdrawals was at least 8 days. In most cases, the concentrations of all three acids were estimated in the same sample of C.S.F. The grand mean is the mean ± standard deviation (No. of estimates) of all the estimates for the particular acid.

	Concentrations in mμg/ml. of C.S.F. of various dogs		
Dog No.	HVA	DOPAC	5-HIAA
I	1,864±220 (7)	200±10 (3)	208±17 (6)
II	2,755±247 (10)	303±60 (7)	302±27 (8)
III	1,677±188 (4)	205±38 (4)	260±35 (3)
IV	1,740±198 (2)	220±17 (3)	230±28 (2)
V	2,263±140 (3)	213±76 (3)	293±23 (3)
VI	2,503±300 (3)	167±21 (3)	327±45 (3)
VII	2,776±285 (3)	270±26 (3)	290±46 (3)
VIII	2,400 (1)	260 (1)	370 (1)
Grand mean	2,298±491 (33)	237±62 (27)	276±52 (29)

TABLE 3

ANALYSIS OF VARIANCE OF THE CONCENTRATIONS OF HOMOVANILLIC ACID 3, 4-DIHYDROXYPHENYLACETIC ACID AND 5-HYDROXYINDOL-3-YLACETIC ACID IN DOG LATERAL VENTRICULAR C.S.F.

Acid	Source of variation	Degrees of freedom	Sum of squares	Variance estimate	Variance ratio	*P*
Homovanillic acid	Between dogs	7	6395909·56	913701·36	17·3	<0·01
	Within dogs	25	1332696·44	53303·86		
3,4-Dihydroxy-phenylacetic acid	Between dogs	7	59742·3275	8534·618	4·07	<0·01
	Within dogs	19	39776·1960	2093·483		
5-Hydroxyindol-3-ylacetic acid	Between dogs	7	56116·093	8016·585	8·8	<0·01
	Within dogs	21	19116·673	910·318		

TABLE 4

EFFECT OF SERIAL SAMPLING OF DOG'S LATERAL VENTRICULAR C.S.F. ON THE CONCENTRATIONS OF HOMOVANILLIC ACID, 3,4-DIHYDROXYPHENYLACETIC ACID AND 5-HYDROXYINDOL-3-YLACETIC ACID IN THE C.S.F. SAMPLES

The concentration at each time is expressed as a percentage of the concentration (mμg/ml. C.S.F.) at zero time. The dogs were maintained under light thiopentone anaesthesia during the experiments with the exception of dog *E* which was conscious throughout. n.d., No estimate obtained.

(*a*) *C.S.F. samples withdrawn at* 1 *hr intervals*

Time of sampling (hr)	Homovanillic acid					3,4-Dihydroxyphenylacetic acid					5-Hydroxyindol-3-ylacetic acid				
	Dog *A*	*B*	*C*	*D*	*E*	Dog *A*	*B*	*C*	*D*	*E*	Dog *A*	*B*	*C*	*D*	*E*
0	100 (1,800)	100 (1,525)	100 (2,400)	100 (2,440)	100 (1,680)	100 (260)	100 (200)	100 (260)	100 (260)	100 (230)	100 (260)	100 (200)	100 (240)	100 (370)	100 (300)
1	99	100	97	99	95	73	75	111	108	73	100	100	100	100	94
2	96	98	104	99	n.d.	85	80	80	100	n.d.	100	100	88	104	n.d.
3	98	10[illegible]	106	99	90	115	85	96	100	65	100	100	98	104	89
4	99	100	108	94	81	85	75	92	108	84	100	105	100	104	91
5	99	90	105	96	85	87	70	77	108	79	100	105	100	97	89
6				94					96						

(*b*) *C.S.F. samples withdrawn at* 0·5 *hr intervals*

Time of sampling (hr)	Homovanillic acid			3,4-Dihydroxyphenylacetic acid			5-Hydroxyindol-3-ylacetic acid		
	Dog *F*	*G*	*H*	Dog *F*	*G*	*H*	Dog *F*	*G*	*H*
0	100 (2,440)	100 (2,880)	100 (1,880)	100 (300)	100 (210)	100 (290)	100 (310)	100 (320)	100 (240)
0·5	94	106	91	73	95	86	103	100	100
1	93	96	93	47	86	83	n.d.	97	90
1·5	100	90	94	40	72	93	100	97	92
2	100	89	85	n.d.	81	72	103	90	92
2·5	87	75	91	40	71	70	110	90	100
3	86	74	91	40	71	72	110	81	100
3·5	86	n.d.	89	37	71	76	100	80	92

(*c*) *C.S.F. samples withdrawn at* 0·25 *hr intervals*

Dog	Time of sampling (hr)	0	0·25	0·5	0·75	1	1·25	1·5	1·75	2	2·25	2·5	2·75	3	3·25
I	Homovanillic acid	100 (1,900)	99	100	101	96	89	89	33	62	38	50	42	50	50
	5-Hydroxyindol-3-ylacetic acid	100 (190)	n.d.	84	89	89	79	100	58	63	47	58	79	53	52
K	Homovanillic acid	100 (2,100)	94	81	90	83	71	57	72	52	48	50	52		
	5-Hydroxyindol-3-ylacetic acid	100 (240)	104	125	94	109	55	109	84	75	63	63	63		

mean concentration for HVA was 2,298 mμg/ml. of C.S.F., with quite a wide range of 1,677–2,776 mμg/ml. for the mean estimates for the eight dogs. The mean concentration of DOPAC was 237 mμg/ml. of C.S.F., which is about one tenth of that for HVA. Somewhat similar concentrations to those of DOPAC were found for 5-HIAA, the grand mean concentration being a little higher, 276 mμg/ml. of C.S.F.

An analysis of variance (Table 3) showed that the variation in acid concentrations for HVA, DOPAC and 5-HIAA between the dogs was significantly greater ($P<0.01$) than that between samples obtained from the same dog on different days. Thus, in the planning of further experiments account was taken of this and the " between dogs " source of variation eliminated by using each dog as its own control.

Repeated sampling of C.S.F. with intervals of 1, 0.5 or 0.25 hr

Table 4 shows the concentrations of HVA, DOPAC and 5-HIAA in successive 0.5 ml. samples of ventricular C.S.F. withdrawn at intervals of 1, 0.5 and 0.25 hr respectively over

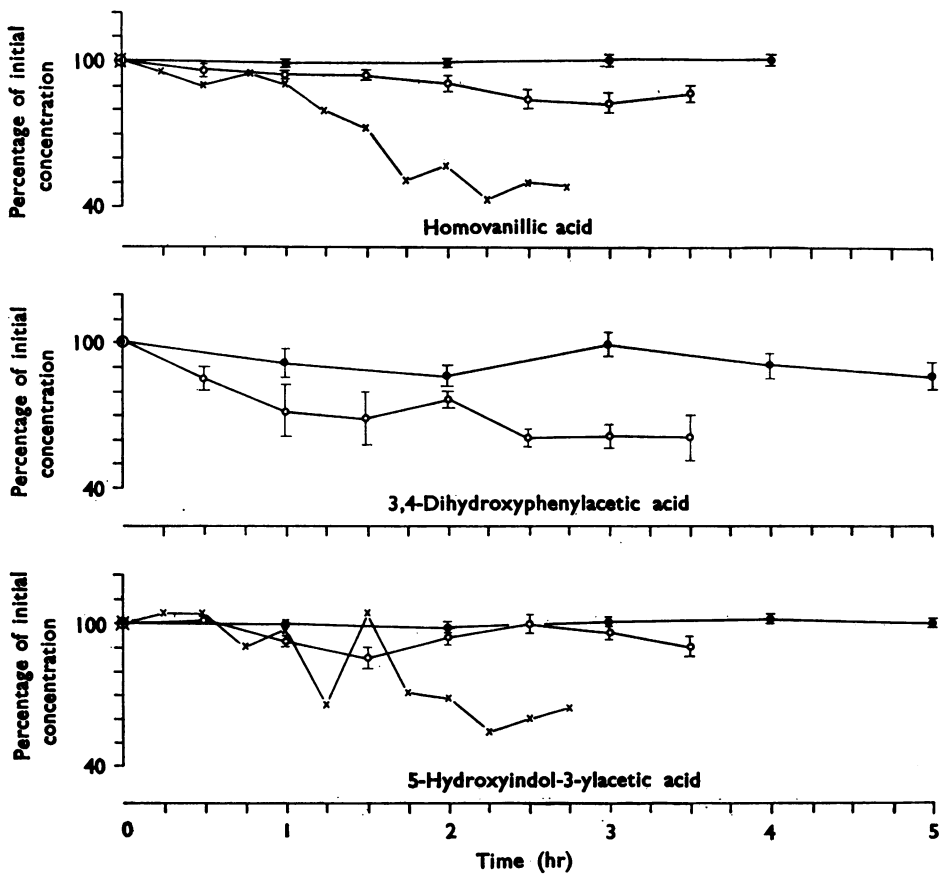


Fig. 5. Effect of repeated sampling on the mean concentrations of phenolic acids in dog C.S.F. Samples, 0.5 ml. volume, of later ventricular C.S.F. were withdrawn at intervals of 1 hr (●——●), 0.5 hr (○——○) and 0.25 hr (×——×). Vertical lines indicate standard errors. Dogs were kept under thiopentone anaesthesia throughout the experiment.

a period of 3–5 hr. All experiments were performed on dogs under light thiopentone anaesthesia, except one which was done on a conscious animal (dog *E*, Table 4). The conscious dog invariably became restless, making the removal of the C.S.F. samples difficult. It is evident that the anaesthetic did not significantly alter the initial concentrations of the acids, but there tended to be a bigger fall in the conscious animal over successive samples possibly because of the technical difficulties.

The summarized results are shown graphically in Fig. 5. With one hourly sampling the concentrations of the acids were well maintained throughout the experiment. With more frequent sampling at 0.5 hr intervals there was some tendency for the acid concentrations to show a progressive decrease, this being most evident in the case of DOPAC, which fell to a level as low as 40% of its initial concentration in one of the dogs.

With sampling at intervals of 0.25 hr, the concentrations of the two acids estimated—HVA and 5-HIAA—seemed to be reasonably well maintained within the first hour of sampling, but they showed a marked decrease between 1 and 2 hr and, in the case of 5-HIAA, considerable fluctuations. It was also noted that with such frequent withdrawals of C.S.F. it became much more difficult to obtain an easy flow of the fluid after about one hr.

DISCUSSION

By a combination of a solvent extraction procedure with spectrophotofluorimetry it has been found possible to measure HVA, DOPAC and 5-HIAA in the same 0.5 ml. sample of ventricular C.S.F. from the dog, allowing the study of parameters of the metabolic pathways of two cerebral amines, dopamine and 5-hydroxytryptamine. We had hoped to include at least one parameter from noradrenaline metabolism as well, but the results of the chromatographic experiments did not give any evidence for the presence of either VMA or DOMAC in the lateral ventricular C.S.F. of the dog. So far, the results of our qualitative work have been indicative of the presence of HVA, DOPAC and 5-HIAA in the C.S.F. No evidence was obtained from the occurrence of *p*-hydroxyphenylacetic acid or *p*-hydroxymandelic acid, which might have been present as a result of cerebral metabolism of tyramine and octopamine respectively. Likewise we failed to detect any 4-hydroxy-3-methoxyphenylethanol or 5-hydroxytryptophol, possible metabolites of dopamine and 5-hydroxytryptamine.

When 0.5 ml. portions of lateral ventricular C.S.F. were withdrawn from a dog at intervals of 1 hr the concentrations of HVA, DOPAC and 5-HIAA in the samples remained virtually constant throughout the 5-hr period of sampling. The volume of the C.S.F. sample withdrawn each time is small compared with the total volume of C.S.F., which is probably of the order of 12 ml. (Davson, 1956), but it is in the region of 25% of the volume of one lateral ventricle. There is, however, good reason to believe that the withdrawn amount of fluid can be easily regenerated within the interval of 1 hr between sampling (Davson, 1956). The constancy of the concentrations of the endogenous acids must be maintained by replacement of the acids removed in a sample, by the entry of the acids from outside the ventricular system to re-establish equilibrium concentrations. There is good evidence that such organic acids do not pass readily from blood to brain (Carlsson & Hillarp, 1962) or from blood to C.S.F. (Guldberg & Yates, 1968) and the most likely source is therefore from brain metabolism.

The concentrations of the acids were not so well maintained if C.S.F. was sampled more frequently. With 0.5 hr intervals significant alterations in concentrations were evident only in the case of DOPAC, while at 0.25 hr intervals they became evident in the case of the other two acids also. It therefore appears that at least 0.5 hr is necessary for the re-equilibration of the acids to occur.

The concentration of HVA, about 2 μg/ml., found in the C.S.F. of the lateral ventricle of the dog was about 10 times higher than that of DOPAC or 5-HIAA. A relatively high concentration of HVA is also present in the caudate nucleus of this species (Laverty & Sharman, 1965). The caudate nucleus bulges into the lateral ventricle and makes up most of its lateral wall through which the acid could gain access to the C.S.F. We have found the concentration of DOPAC in the caudate nucleus of the dog to be about one-tenth of that of HVA and, as already pointed out, a similar ratio for the acids occurs in the C.S.F. It seems then that the levels of HVA and DOPAC in the ventricular C.S.F. are directly related to the concentrations of the acids in the caudate nucleus. The concentration of these two acids in the caudate nucleus is about 6 times higher than in the lateral ventricular C.S.F., but the corresponding ratio for 5-HIAA is much less, being about one. If it is postulated that there is no difference in the mechanism of access of the three acids from brain tissue into C.S.F., it is evident that the caudate nucleus cannot be the main source of 5-HIAA in the samples of C.S.F. taken from the lateral ventricle. Other areas proximal to the ventricular system which contain relatively higher concentrations of 5-HIAA will contribute. One such area may well be the amygdala, which contains high concentrations of 5-hydroxytryptamine (Bogdanski, Weissbach & Udenfriend, 1957) and which is adjacent to the inferior horn of the lateral ventricle. It is also possible that some of the 5-HIAA in the lateral ventricular C.S.F. is derived by diffusion from the third ventricle, which is bounded by areas containing high 5-HIAA concentrations such as the hypothalamus.

The technique of repeated sampling of lateral ventricular C.S.F. at short (1 hr) or longer intervals under conditions giving constant levels of HVA, DOPAC and 5-HIAA in the samples from normal animals has been used to follow, over a period of time, drug-induced alterations in the concentrations of those acids in the C.S.F. Some such studies are described in the following paper (Guldberg & Yates, 1968) in which the trend in any such alteration has been compared with that reported for the acid concentrations in the brain itself. The establishment of a correlation between C.S.F. concentrations and brain concentrations of the acids might allow a new approach to the investigation of the metabolism of dopamine and 5-hydroxytryptamine in brain without recourse to brain analysis and would therefore be applicable to man as well as to animals.

SUMMARY

1. A method for repeated sampling of lateral ventricular cerebrospinal fluid (C.S.F.) in chronic experiments on dogs has been described.

2. A technically simple procedure adapted from existing methods for the estimation of homovanillic acid (HVA), 3-4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindol-3-ylacetic acid (5-HIAA), in a single 0.5 ml. sample of lateral ventricular C.S.F. from the dog is described.

3. Lateral ventricular C.S.F. from the dog was found to contain HVA, DOPAC and 5-HIAA, the mean concentrations being 2.3, 0.24 and 0.28 μg/ml. of C.S.F. respectively.

4. Results from thin-layer chromatography provided confirmatory evidence for the presence of HVA, DOPAC and 5-HIAA in lateral ventricular C.S.F. of the dog. Vanillinmandelic acid, 3,4-dihydroxymandelic acid, *p*-hydroxymandelic acid, *p*-hydroxyphenylacetic acid, 4-hydroxy-3-methoxyphenylethanol and 5-hydroxytryptophol, all of which are possible metabolites of biologically occurring amines, were not detected.

5. The withdrawal of 0.5 ml. of ventricular C.S.F. from the dog repeated at intervals of 1 hr for up to 5 hr did not alter the concentrations of HVA, DOPAC and 5-HIAA in the successive samples. More frequent sampling at intervals of 0.5 hr caused a decrease in the concentrations of DOPAC and 0.25 hourly sampling led to a significant fall in the concentrations of all three acids in the course of the 3.5 hr period during which samples were withdrawn.

6. The use of serial sampling of ventricular C.S.F. as a method for studying drug effects and the value of estimations of certain substances in the C.S.F. as indicators of some aspects of brain metabolism are suggested.

We thank Professor W. L. M. Perry for his invaluable help and advice in this work.

REFERENCES

ANDÉN, N.-E., ROOS, B.-E. & WERDINIUS, B. (1963a). The occurrence of homovanillic acid in brain and cerebrospinal fluid and its determination by a fluorometric method. *Life Sci.*, **2**, 448–458.

ANDÉN, N.-E., ROOS, B.-E. & WERDINIUS, B. (1963b). On the occurrence of homovanillic acid and 3-methoxy-4-hydroxymandelic acid in human cerebrospinal fluid. *Experientia*, **19**, 359–360.

ANDÉN, N.-E., ROOS, B.-E. & WERDINIUS, B. (1964). Effects of chlorpromazine, haloperidol and reserpine on the levels of phenolic acids in rabbit corpus striatum. *Life Sci.*, **3**, 149–158.

ASHCROFT, G. W. & SHARMAN, D. F. (1960). 5-Hydroxyindoles in human cerebrospinal fluids. *Nature, Lond.*, **186**, 1050—1051.

ASHCROFT, G. W. & SHARMAN, D. F. (1962). Drug-induced changes in the concentration of 5-OR indolyl compounds in cerebrospinal fluid and caudate nucleus. *Br. J. Pharmac. Chemother.*, **19**, 153–160.

BERNHEIMER, H., BIRKMAYER, W. & HORNYKIEWICZ, O. (1966). Homovanillinsäure in liquor cerebrospinalis: Untersuchungen beim Parkinson-Syndrom und Anderen Erkrankungen des ZNS. *Klin. Wschr.*, **78**, 417–419.

BOGDANSKI, D. F., WEISSBACH, H. & UDENFRIEND, S. (1957). The distribution of serotonin, 5-hydroxytryptophan decarboxylase and monoamine oxidase in brain. *J. Neurochem.*, **1**, 272–278.

CARLSSON, A. & HILLARP, N. A. (1962). Formation of phenolic acids in brain after administration of 3,4-dihydroxyphenylalanine. *Acta physiol. scand.*, **55**, 95–100.

DAVSON, H. (1956). *Physiology of the Ocular and Cerebrospinal Fluid.* London: Churchill.

GOTTFRIES, C. G., ROSENGREN, A. M. & ROSENGREN, E. (1965). The occurrence of homovanillic acid in human brain. *Acta pharmac. tox.*, **23**, 36–40.

GULDBERG, H. C., ASHCROFT, G. W. & CRAWFORD, T. B. B. (1966). Concentrations of 5-hydroxyindolylacetic acid and homovanillic acid in the cerebrospinal fluid of the dog before and during treatment with probenecid. *Life Sci.*, **5**, 1571–1575.

GULDBERG, H. C. & YATES, C. M. (1968). Some studies of the effects of chlorpromazine, reserpine and dihydroxyphenylalanine on the concentrations of homovanillic acid, 3,4-dihydroxyphenylacetic acid and 5-hydroxyindol-3-ylacetic acid in ventricular cerebrospinal fluid of the dog using the technique of repeated sampling of the cerebrospinal fluid. *Br. J. Pharmac. Chemother.*, **33**, 457–471

JUORIO, A. V., SHARMAN, D. F. & TRAJKOV, T. (1966). The effect of drugs on the homovanillic acid content of the corpus striatum of some rodents. *Br. J. Pharmac. Chemother.*, **26**, 385–392.

LAVERTY, R. & SHARMAN, D. F. (1965). Modification by drugs of the metabolism of 3,4-dihydroxyphenylethylamine, noradrenaline and 5-hydroxytryptamine in the brain. *Br. J. Pharmac. Chemother.*, **24**, 759–772.

MANUILOV, I. A. (1958). Technique for perfusion of brain ventricles in dogs under chronic experimental conditions. *Sechenov physiol. J. U.S.S.R.*, **44**, 458–462.

RANDERATH, K. (1963). *Thin-layer Chromatography*, p. 45. Weinheim/Bergstr.: Verlag Chemie GmbH; New York and London: Academic Press.

ROSENGREN, E. (1960). On the role of monoamine oxidase for the inactivation of dopamine in brain. *Acta physiol. scand.*, **49**, 370–375.

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

WEIL-MALHERBE, H. (1959). The fluorimetric estimation of catechol compounds by the ethylenediamine condensation method. *Pharmac. Rev.*, **11**, 278–288.