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EFFECT OF INTRAVENOUSLY ADMINISTERED PROBENECID IN HUMANS ON THE LEVELS OF 5-HYDROXYINDOLEACETIC ACID, HOMOVANILLIC ACID AND 3-METHOXY-4-HYDROXY-PHENYLGLYCOL IN CEREBROSPINAL FLUID*

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Abstract—After isolation and separation on columns of Sephadex G10, 5-hydroxyindoleacetic acid, homovanillic acid and 3-methoxy-4-hydroxyphenylglycol from human lumbar cerebrospinal fluid was measured fluorimetrically. Details about fluorimetry of the glycol are given. The effect of intravenously administered probenecid on the levels of the three compounds in lumbar CSF was investigated as a function of time. The acids, 5-hydroxyindoleacetic acid and homovanillic acid, accumulated not earlier than 6 hr after the administration of the drug. The noradrenaline metabolite, 3-methoxy-4-hydroxyphenylglycol, did not accumulate under the influence of probenecid. The lag phase of about 4 hr before accumulation of the acids in lumbar CSF occurs, might reflect on the transport time along the spinal cord of the acids from the cisternal- to the lumbar region.

THE TURNOVER rate of noradrenaline (NA) in the spinal cord of the rat is increased by electrical stimulation of the medulla oblongata.[1] Electrical stimulation of the midbrain raphe of the rat induced a raising of the levels of telencephalic 5-hydroxyindoleacetic acid (5-HIAA).[2] These and other experimental data indicate that the functional state of NA- and 5-hydroxytryptamine (5-HT) containing neurons is correlated with the chemical turnover rate of these amines (Neff and Tozer,[3] Carlsson[4] and Andén *et al.*[5]).

In rats an estimation of 5-HT turnover rate can be executed by measuring the rate of accumulation of 5-HIAA in the brain by a maximally effective dosage of probenecid.[3] The turnover rate of 5-HT determined after treating of rats with probenecid corresponds rather well with values of the turnover rate of 5-HT measured with other methods.

Probenecid inhibits the efflux of 5-HIAA not only from the brains of rats, rabbits and mice but also from cerebrospinal fluid (CSF) of dogs[6] and humans.[7, 8] In humans direct measuring of brain metabolism of NA, Dopamine (DA), and 5-HT is not possible, but determining the levels of the main metabolites of these amines, 3-methoxy-4-hydroxy phenyleneglycol (MOPEG), Homovanillic acid (HVA) and 5-HIAA, respectively, in human CSF might reveal information concerning brain metabolism.

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Guldberg[9] found, that the HVA and 5-HIAA concentrations in the lateral ventricular CSF of dogs correspond well with the content of these compounds in the caudate nucleus. Intravenous injection in humans of L-DOPA-^{14}C, a precursor of brain DA, caused an accumulation of HVA-^{14}C in the cisternal and the lumbar CSF.[10] Only traces of radioactivity were found in the CSF after HVA-^{3}H injection, indicating that HVA is not transferred direct from the blood to CSF.[10,11] It is therefore likely that the HVA-^{14}C observed in CSF is derived from the brain.[10] Lumbar CSF of patients suffering from Parkinson's disease contains less HVA than normal CSF.[12, 13] This might reflect on the lower DA concentrations in the brains of Parkinson patients. *p*-Chlorophenylalanine lowers the concentrations of 5-HT and 5-HIAA of the brain, as well as of 5-HIAA in the CSF.[14]

These findings supported the idea, that there is a close relationship between brain metabolism of 5-HT and DA and the CSF levels of 5-HIAA and HVA. If this is so, information concerning the turnover rates of 5-HT and DA in the brain might be obtained by inhibiting the active transport of 5-HIAA and HVA out of the CSF by probenecid, so that accumulation of these acids occurs. In the studies in humans,[7, 8] probenecid was administered orally for 3 days. The experiments do not give any information about the rate of accumulation of the acids in CSF, because in these experiments new steady state levels of 5-HIAA and HVA might have been reached.

In this paper we shall describe the effect of intravenously administered probenecid on the levels of HVA and 5-HIAA in CSF as a function of the time.

Large enough samples of lumbar CSF of humans for simultaneous determination of HVA, 5-HIAA and MOPEG are hardly obtainable. Therefore we devised a method for the determination of these compounds in the same sample of CSF. The fluorimetrical methods used in this study of HVA and 5-HIAA are published elsewhere.[15,16] Details of a method for the determination of MOPEG are published in this paper. Schanberg *et al.*[17] determined MOPEG and the sulfate conjugate of MOPEG, after enzymatic hydrolysis, by the gaschromatographic method of Wilk *et al.*[18] Taylor[19] oxidized MOPEG to a fluorescent product, probably the dimer, but could not detect MOPEG in the brain of the rat with his method of isolation and fluorophore formation.

Isolation and separation of MOPEG, HVA and 5-HIAA of lumbar CSF was performed on columns of Sephadex G10. The method was applied for the investigation of the rate of accumulation of MOPEG, HVA and 5-HIAA after intravenous administration of 1 g probenecid in humans.

MATERIALS AND METHODS

Homovanillic acid, 5-Hydroxyindoleacetic acid (Fluka); 3-Methoxy-4-hydroxyphenyleneglycol-piperazine salt (Calbiochem); Sephadex G10 (Pharmacia) and Probenecid* (Merck, Sharp and Dohme) were used.

Isolation of MOPEG, HVA and 5-HIAA

Sephadex G 10 columns (diameter 1·6 cm, height 5 cm) were washed with 20 ml bidistillated water before application of the sample. Lumbar CSF (3 or 4 ml) was adjusted to a pH between 2 and 3 with concentrated formic acid and applied to a

* Probenecid ampoules were generously provided by the manufacturers.

column of Sephadex G10. After the sample had passed through it, the column was washed with 6 ml 0·01 N acetic acid to remove salts and proteins (flow rate 30 ml/hr).

MOPEG was eluted with 4 ml 0·01 N acetic acid, 5 ml water and 3 ml 0·5 M Na_2HPO_4-NaH_2PO_4 buffer (pH 8·5). Because of the void volume of the column the eluted MOPEG was dissolved only in the acetic acid–water mixture. HVA was eluted by 6 ml of the buffer solution and 4 ml water, so that the whole phosphate buffer fraction was obtained (10 ml).

5-HIAA was eluted with 8 ml 0·01 N ammonia. The composition of this eluate was about 4 ml water and 4 ml of the ammonia solution.

Fluorometric analysis

The development of the fluorophore of MOPEG was obtained after reaction with ethylenediamine and $K_3Fe(CN)_6$. In Table 1 the composition of the reaction mixture and the order of addition of reagents are given. The HVA fluorophore was developed according to the method of Gerbode and Bowers.[14] Details are described elsewhere.

TABLE 1. DEVELOPMENT OF A FLUOROPHORE OF 3-METHOXY-4-HYDROXYPHENYLENE GLYCOL

Order of addition of reagents		Blank	Sample	Sample + Standard
Eluate	ml	2·0	2·0	2·0
MOPEG (1 μg/ml)	ml	—	—	0·1
H_2O	ml	0·1	0·1	—
$K_3Fe(CN)_6$, 0·2%	ml	—	0·1	0·1
Cystein, 1%	ml	0·1	—	—
Ethylenediamine reagent*	ml	1·0	1·0	1·0
Water-bath, 70°, 15 min		+	+	+
$K_3Fe(CN)_6$, 0·2%	ml	0·1	—	—
Cystein, 1%	ml	—	0·1	0·1

Excitation/Fluorescence 400/500 nm.

* Ethylene diamine reagent: equal parts of ethylenediamine and a mixture of 4 N NH_4Cl–4 N NH_4OH, 1:9 (v/v).

The fluorophore of 5-HIAA was developed after a reaction with *o*-phthaldialdehyde in 6 N HCl. The method was based on our earlier experience with this method[16] (Table 2).

Probenecid test

Within 20 min 1 g probenecid, dissolved in 250 ml isotonic solution, was infused in the antecubital vein.

From each patient two samples of 5–10 ml lumbar CSF were obtained by a routine puncture. One sample was obtained 2 days before the administration of probenecid. The other lumbar punctures were made 2, 4, 6, 8, 10 or 12 hr after the administration of the drug. Immediately after the puncture the CSF samples were placed in a freezer (−20°). Both the samples of one patient were analyzed together within 5 days after collection.

TABLE 2. DEVELOPMENT OF A FLUOROPHORE OF 5-HYDROXYINDOLEACETIC ACID

Order of addition of various reagents		Blank	Sample	Sample + Standard
Eluate	ml	1·0	1·0	1·0
5-HIAA, 1 μg/ml	ml	—	—	0·1
H_2O	ml	0·1	0·1	—
Cystein, 1%	ml	—	0·1	0·1
Na-periodate, 0·02%	ml	0·1	—	—
Concentrated HCl	ml	2·0	—	—
90 min, room temp.		+	—	—
Concentrated HCl	ml	—	2·0	2·0
Cystein, 1%	ml	0·1	—	—
Na-periodate, 0·02%	ml	—	0·1	0·1
o-Phthaldialdehyde, 0·1% (in absolute methanol)	ml	0·1	0·1	0·1
10 min, boiling water		+	+	+

After cooling, the fluorescence at wavelength 365/490 nm excitation/fluorescence, respectively was read.

The donors of the CSF were 22 patients of the Psychiatric Clinic of Groningen University; 15 patients were suffering from depression (eight from vital depression and seven from endogenous depression). The other seven belonged to a non-depressive group.

RESULTS

Isolation of the compounds

The isolation procedure produces almost complete separation of MOPEG and HVA. The recovery of the compounds in their fractions are 85 and 72 per cent respectively. Some MOPEG contaminated the HVA fraction (about 10 per cent) and some of the MOPEG was eluted before the desalting of the CSF was complete. The binding of MOPEG to the Sephadex is not very strong. This is the reason why we had to use rather large columns for the isolation and separation procedure.

About 20 per cent of the HVA was eluted in the 5-HIAA fraction. Without any difficulty it is possible to elute all HVA with the buffer, but then it is necessary to use 15–20 ml of the phosphate buffer. Because of the nonlinearity of the fluorometrical determination of HVA we prefer this incomplete recovery in 10 ml, to a complete recovery of HVA in a fraction of about 20 ml.

All 5-HIAA was recovered in the ammonia-water mixture. By using Sephadex G10 columns for the isolation of 5-HIAA from human urine, Contractor,[20] too, obtained high recoveries.

Fluorometry of MOPEG

The spectral characteristics of the fluorescing compound formed from MOPEG, ethylenediamine and $K_3Fe(CN)_6$ are presented in Fig. 1. In the case of MOPEG it was necessary to use an oxidant; such an oxidant can be omitted in the fluorometric methods of catechol compounds based on ethylenediamine condensation. By using a

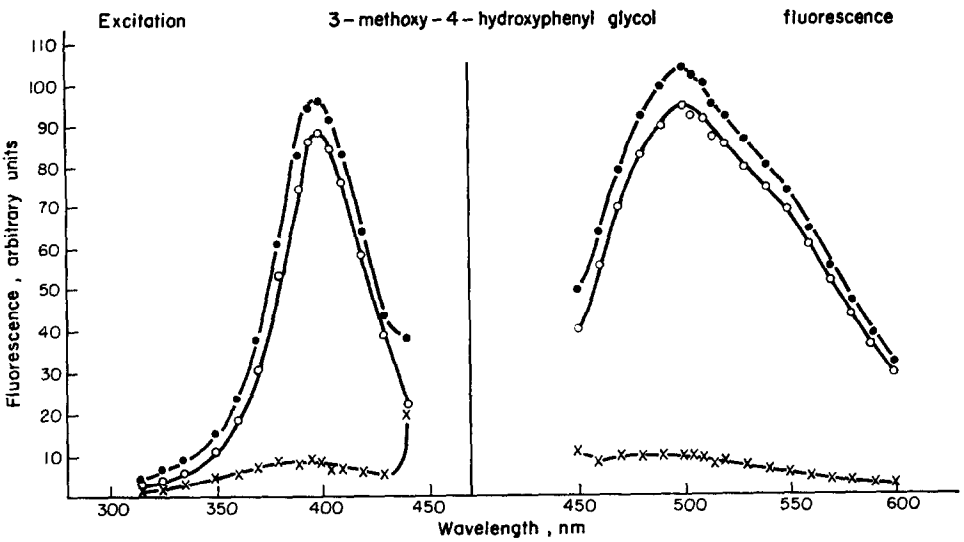

FIG. 1. Excitation and fluorescence spectra of authentic MOPEG after reaction with ethylenediamine and $K_3Fe(CN)_6$. –·–·– MOPEG, –×–×– blank, –○–○– difference spectra, obtained by subtracting the spectra of the blank from those of MOPEG.

mixture of ammonium chloride and ammonia, optimal conditions were obtained rather than with NH_4Cl solutions used with catechol compounds. The mixture used in our procedure was chosen after variations of the quantities added of NH_4Cl and ethylenediamine. The concentration of $K_3Fe(CN)_6$ was varied from 0·01 to 0·5%. The best results were obtained with 0·1 or 0·2% $K_3Fe(CN)_6$ solutions. Incubation time and temperature were optimized too.

By adding cysteine or omitting the oxidant a blank was obtained corresponding to about 0·02 μg MOPEG/ml standard solution. With CSF eluates exactly the same blanks are revealed by preventing the oxidation in the two ways described.

It is necessary to determine a blank for each CSF eluate, because of the low concentrations of MOPEG. Figure 2 gives the relationship between the fluorescence measured and the quantity of MOPEG (dissolved in 2 ml water). This relationship is linear; the procedure is highly reproducible as can be seen from the variations of each of the triplicate determination, given in the Figure.

Under the reaction conditions of the fluorophore formation of MOPEG some related compounds are converted into an interfering fluorophore, but the sensitivity to the detection of most of the compounds is far less. Of 31 catechol-, guaiacol- and indol-derivatives tested DOPA, normetanephrine, metanephrine adrenaline and guaiacol interfere considerably. By the isolation procedure the two non-amine derivatives were eliminated. In CSF the concentration of the amines is too low for disturbance.

Probenecid experiment

The ratios of the concentration of HVA, 5-HIAA and MOPEG after and before probenecid administration (A/B) as a function of time are represented in Figs. 3–5.

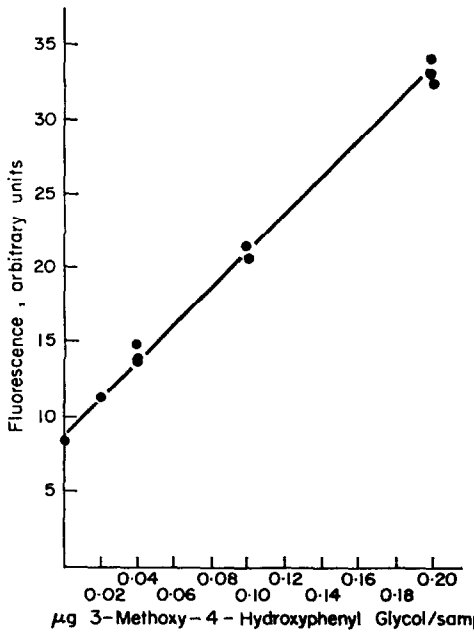

FIG. 2. Relationship between quantity of MOPEG and fluorescence. Each quantity of MOPEG was measured in triplicate. In some cases two or three measurements had the same value.

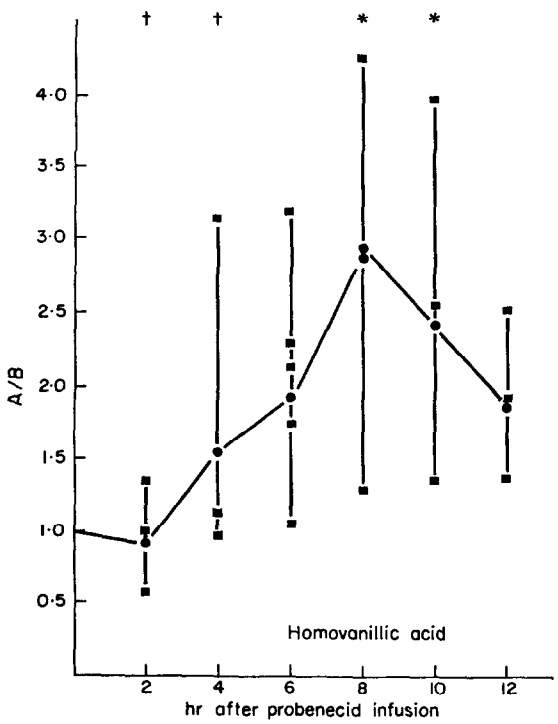

FIG. 3. Accumulation of HVA in human lumbar CSF as a function of time, after intravenous administration of 1 g probenecid. A/B: ratio of concentration of HVA after and before probenecid treatment. Each point of the curve is the ratio A/B in one patient (* and †: $P < 0{\cdot}025$).

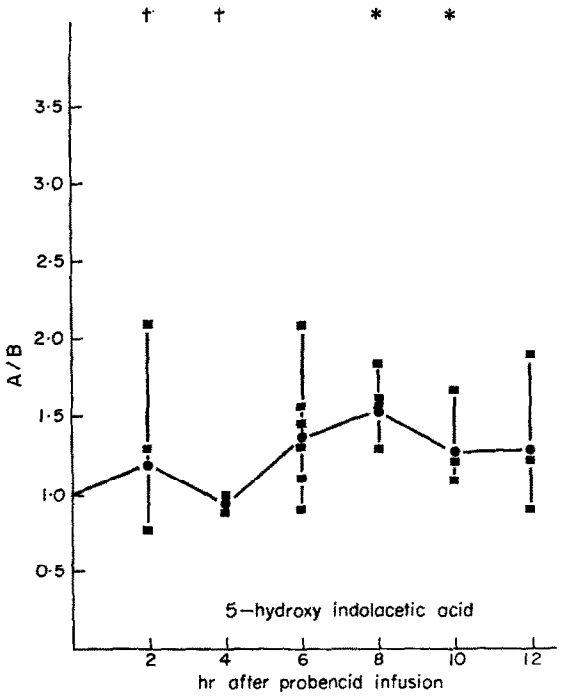

FIG. 4. Accumulation of 5-HIAA in human lumbar CSF as a function of time (see Fig. 3). (* and †: $P < 0.050$).

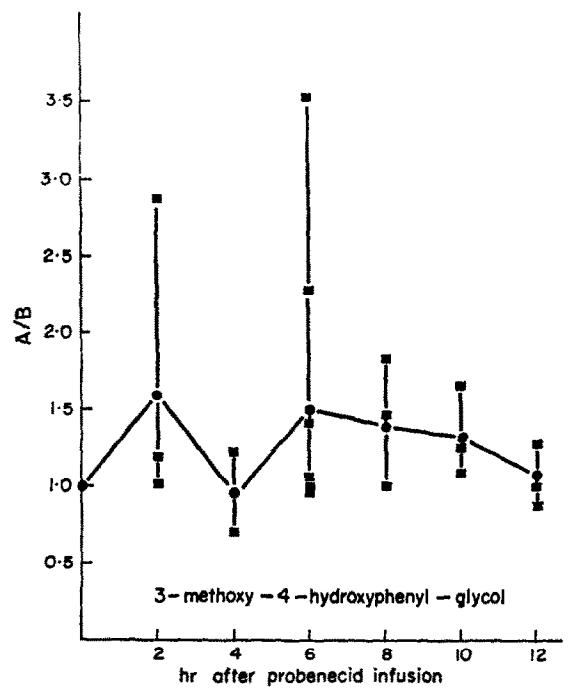

FIG. 5. Effect of probenecid on the concentration of MOPEG in human lumbar CSF (for details see Fig. 3). A/B of the 2- and 4-hr samples compared to ratios of the other samples do not differ ($P > 0.15$).

From Figs. 3 and 4 we may conclude that both the acids, HVA and 5-HIAA, accumulate after the intravenous administration of 1 g probenecid. Comparing the ratios A/B of HVA in the 2- and 4-hr samples with the 8- and 10-hr samples, we see that the accumulation was statistically significant ($P < 0.025$ one tailed Mann Whitney test[21]). The ratios A/B of 5-HIAA in the 2 and 4 hr samples differed significantly from the 8- and 10-hr CSF samples ($P < 0.05$).

From our data we may conclude, that there is no accumulation of HVA and 5-HIAA within 4 hr after the intravenous administration of the drug.

MOPEG did not accumulate to any statistical significance (Fig. 5). This finding is in agreement with studies among a group of patients receiving orally 3 g probenecid for 3 days.[8]

The levels of the compounds in lumbar CSF, given with S.E.M. of the whole group of patients before drug administration were:

MOPEG: 0.029 ± 0.015 μg/ml (number of values 21).
HVA: 0.043 ± 0.013 μg/ml (19).
5-HIAA: 0.041 ± 0.022 μg/ml (21).

DISCUSSION

Biochemical methods

Isolation and separation of MOPEG, HVA and 5-HIAA by means of columns of Sephadex G10 allows one to isolate the 3 compounds in one step. MOPEG was eluted in a 12 ml fraction, HVA in a fraction of 10 ml and 5-HIAA in 8 ml. By using more elution fluid it is possible to recover 95–100 per cent of the three compounds. Kariya and Aprison[22] described a method for the extraction of NA, DA, 5-HT and acetyl choline from the brain of one rat. We applied this method—extraction of brain tissue with a mixture of formic acid and acetone—for the isolation of MOPEG, HVA and 5-HIAA from the brain of one rat, and analysed the compounds with the method described in this paper. HVA and 5-HIAA levels in the brain of the rat, 0·19 and 0·47 μg/g wet wt. of brain tissue respectively squared with literature values. The content of MOPEG in the brain of the rat found in this study (0·37 μg/g), however, was higher than the value found by Schanberg *et al.*[17] possibly because of interference of the catecholamines. Our method is therefore more suitable for CSF than for the determination of MOPEG in the rat brain. In this study we have not made an exhaustive investigation to establish whether the MOPEG obtained after isolation from the CSF is contaminated with interfering substances. Fluorescence spectra, however, agree very well with the spectra of authentic MOPEG.

During the course of this investigation an alternative fluorophore formation from MOPEG has been described.[19] This fluorophore was formed by the oxidation of MOPEG by iodine. Our system is slightly more sensitive, because the blanks in the iodine method correspond to 0·1 μg; the blanks in our method correspond to 0·06 μg MOPEG. With the method described in this paper we found a linear relationship between the concentration and the fluorescence in the concentration range of 0·005 to 0·1 μg MOPEG per ml of the sample.

Probenecid test

Within 4 hr after the intravenous administration of 1 g probenecid there was no accumulation of HVA and 5-HIAA in the lumbar CSF (Figs. 3 and 4). The reason for

this lag phase is not clear from our experiments but some points should be taken into consideration:

The penetration of probenecid into the lumbar region might be very slow, so that it took several hours before the concentration in the lumbar regions is high enough to be effective with respect to the acid transport. Although this possibility cannot be excluded from our experiments, there might be another reason for the lag phase we found. Assuming that probenecid penetrates the lumbar region shortly after intravenous administration, then our experiments suggest that there is no probenecid sensitive transport mechanism in the lumbar sac. Inhibition of a transport mechanism for HVA and 5-HIAA in the lumbar sac would give rise to an accumulation of the acids immediately after probenecid administration. The duration of the lag phase can be explained by comparing our experimental data with those of Pletscher *et al.*[10] After intravenous injection of DOPA-^{14}C Pletscher *et al.*[10] measured the incorporation of ^{14}C into HVA of cisternal and lumbar CSF. The HVA in the cisternal CSF was labelled maximally 2–4 hr after the injection of radioactive DOPA. The highest levels of HVA-^{14}C of lumbar CSF were found 6–8 hr after injection of the precursor. So the time necessary for the transport of radioactive HVA from the cisternal to the lumbar region might be about 4 hr. This time is in accordance with the time interval found in this study between probenecid administration and the appearance of accumulation of HVA and 5-HIAA. This also means that the accumulated acids are transported from brain areas after inhibition of their efflux through the choroide plexus.[6] The results of this study might contribute towards the significance of the concentration of HVA and 5-HIAA in lumbar CSF in relation to brain metabolism of DA and 5-HT. Experiments are now in progress to study the effects of higher doses of probenecid on CSF concentrations of HVA and 5-HIAA.

We could not detect any accumulation of MOPEG in lumbar CSF after administration of probenecid to humans. The reason for the failure of probenecid to inhibit the transport of MOPEG out of the CSF might be the molecular differences of probenecid, HVA and 5-HIAA as compared to MOPEG. The first three compounds are carboxylic acids, MOPEG is a phenolic acid and is not ionized as completely as the carboxylic acids at physiological pH. The pK value of MOPEG is not known to us but the analogous compound guaiacol has a pK value of 7·0. It is likely that the transport of MOPEG out of the brain or CSF, if it does occur at all, will have a different mechanism than HVA and 5-HIAA, in this case a not probenecid sensitive mechanism. At present we are investigating the effect of probenecid on the levels of the sulphate conjugate of MOPEG in human and canine lumbar CSF and in the brains of rats.

Now we come back to the considerations mentioned in the Introduction. Is it possible to obtain information concerning the turnover rate of biogenic amines of human brain by measuring the accumulation rate of the main catabolites of amines in the lumbar CSF? For a positive answer one should take several points into consideration as: e.g. the rate of transport of HVA and 5-HIAA along the spinal cord, the degree of inhibition of the transport of HVA and 5-HIAA out of the brain and CSF by the intravenously administered probenecid, the rate of CSF production, total CSF volume, fraction of the total amount of the acids of the brain transported to the lumbar sac. If these variable parameters are equally spread over the groups of patients one intends to compare, the accumulation rate of the acids after probenecid treatment might have real significance in studies on turnover of biogenic amines in the human

brain. In that case the probenecid test described will be of importance for studies concerning the role of 5-HT and DA in mental disorders.

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