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MEPACRINE METABOLISM: AN EXAMINATION OF MOUSE LIVER FOR POSSIBLE ANTIVIRAL METABOLITES

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Hitherto, in the attempted chemotherapy of the smaller viruses, a few polymeric substances have shown slight activity, but compounds of lower molecular weight have not been effective. Recently, however, Hurst, Melvin, and Peters (1952) showed that mepacrine had a very considerable prophylactic effect on Eastern equine encephalomyelitis in the mouse, but not in the rabbit, guinea-pig, chick, or monkey, and only slightly in the rat. Thus it would appear that either the course of the infection in the mouse is not comparable with that in the other species, or the metabolism or distribution of mepacrine in the mouse is different, and that possibly a very active antiviral metabolite is formed. The latter hypothesis forms the reason for the present work. Staining of mouse liver, spleen, and skin persists for several days after one oral dose. The liver was selected as a convenient organ in which to search for metabolic products, since relatively large amounts of acridine and virus accumulate in it ; furthermore, it is the site at which abundant basiphil particles are found in the cell cytoplasm (Hurst, Snow, and Roberts, 1955).

METHODS

Adsorbents for Chromatography.—Alumina—Grade "0" manufactured by Peter Spence Ltd., Widnes. *Decalso*—Sodium aluminium silicate, manufactured by the Permutit Company, London.

Paper Chromatography of Acridines.—Whatman No. 50 filter paper sheet was immersed in an aqueous 0.1M buffer solution (*p*H 7.3, containing, per litre, potassium dihydrogen phosphate 13.6 g., sodium hydroxide 2.97 g., and trimethylcetyl-ammonium bromide, "Cetavlon," 1.0 g.). The paper was hung up and, when just damp, the solution of the sample was applied in the usual way. The paper sheet was transferred to a chromatographic tank containing filter paper curtains soaked in buffer solution and in ethyl acetate respectively. After the test sheet had been enclosed for 4 hr., 60 ml. of ethyl acetate saturated with the aqueous buffer solution was introduced into the trough, and the development was allowed to proceed during 12 hr. The solvent was allowed to drip off the end of the paper, the edges of which were serrated.

Extraction of Acridines from Liver

To remove mepacrine and any possible metabolites from mouse livers (taken 48 hr. after oral dosing at 500 mg./kg.) with a minimum of fat, protein, etc., the livers (300–400 g.) were disintegrated in a suspension of methanol or *iso*propanol (2 l.) using a Waring blender under a blanket of nitrogen. The suspension filtered readily, and after two further treatments each with 1 l. of the alcohol, only a very small proportion of the mepacrine remained adsorbed on the minced tissue. The combined alcohol extracts were stored in the refrigerator overnight, then filtered. The clear yellow filtrate was evaporated under reduced pressure in nitrogen to a volume of 1 l. This concentrate, on shaking with 1 l. of light petroleum (b.p. 60–80°), formed two distinct yellow layers having a green fluorescence. None of the yellow colour could be extracted from the petroleum solution on shaking with dilute hydrochloric acid. It was estimated colorimetrically that about 38% of the total acridine extracted was retained in the petroleum layer. The analytical work was thus divided into an examination of the aqueous and the petroleum layers.

Countercurrent Distribution of the Aqueous Layer.—The aqueous layer obtained after treatment of 890 g. of the livers as described above was brought to *p*H 6 with phosphoric acid and the volume adjusted to 50 ml. This solution was then subjected to six stages of countercurrent extraction between chloroform (500 ml.) and 0.1M-phosphate buffer (500 ml., *p*H 6). The colorimetric analysis of each stage indicated that there was a partial separation into two fractions in stages 1 and 2 and 3–6 inclusive. The manner in which the stages were combined and worked up is as shown in Table I. For the isolation of the 5–10% of unknown compound from the 480 mg. of impure mepacrine hydrochloride (stages 3–5, Tables I and III), samples were subjected to countercurrent distribution in two different systems: (i) chloroform and 0.1M-citrate buffer (*p*H 5.9), and (ii) ethyl acetate 0.1M-phosphate buffer (*p*H 7.3). The

TABLE I

COUNTERCURRENT EXTRACTION OF WATER-SOLUBLE MEPACRINE METABOLITES

Stage No.	Absorption* Density	Method of Isolation
1	0·7	The aqueous layer brought to *p*H 8, extracted with the chloroform layer, chloroform removed and residue triturated with ether; 21 mg. insoluble solids remained. From the ethereal filtrate 144 mg. of hydrochloride was precipitated.†
2	0·35	
3	0·8	Treatment as above led to an ether soluble product from which 480 mg. of hydrochloride was isolated.
4	0·8	
5	0·4	
6	0·08	The chloroform layer separated and extracted with *p*H 4 citrate buffer. Citrate extract brought to *p*H 8 and transferred to chloroform. After removing chloroform, the solid residue (7 mg.) was insoluble in ether.

* $d_{1\ cm.}$ at 435 mμ.

† The precipitation of hydrochloride is described in Table II, footnote.

results from (i) illustrated in Fig. 1A show a minor component in stages 38–46, with the major component spread over stages 15–35. The dotted line is a theoretical curve for a compound having p=0.59 (where p=fraction of the total distributed in the top layer). The slight divergence of the observed curve from the theoretical is probably due to experimental imperfections, yet the results are such that 5–10% of an impurity having p=0.66 could not be detected.

By using the higher order of separation obtainable from paper chromatography in conjunction with a higher *p*H and ethyl acetate as mobile phase, the sample gave rise to two spots. These were from a secondary component and mepacrine, together with a minute quantity of a third component made visible by ultraviolet irradiation (secondary and tertiary spots travelled 0.25 and 0.17 of the distance traversed by the mepacrine spot; the solvent front was beyond the end of the paper). The proportion of the secondary component was conveniently estimated by bulk countercurrent distribution as in Fig. 1B. Note that the positions of the main and secondary components are reversed in changing from systems (i) to (ii). The amount of the secondary component in stages 2–7 estimated by spectrophotometry at 425 mμ after dilution with 0.1N-hydrochloric acid was approximately 5% of the total fraction.

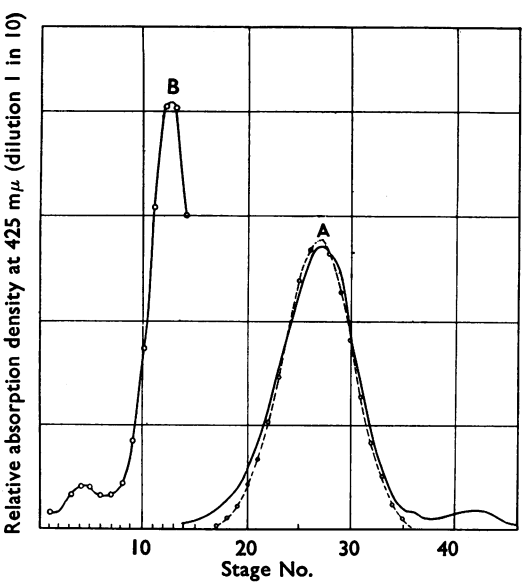

FIG. 1.—Distribution of water-soluble fraction (Table I, stages 3–5) after: (A) 46 transfers between chloroform and 0.1M-citrate buffer, *p*H 5.9 (———). Theoretical curve for a compound having p=0.59 (O – – – O). (B) 13 transfers between ethyl acetate and 0.1M-phosphate buffer, *p*H 7.3 (O———O).

In order to obtain milligram amounts of the secondary component, a fraction comparable to that from stages 3–5 above, prepared from 1,500 g. of livers, was subjected to 19 transfers in the ethyl acetate *p*H 7.3 buffer system. The secondary component separated in stages 1–10 was extracted into chloroform and the solution concentrated under reduced pressure in nitrogen. Then 10 volumes of *n*-hexane were added and after standing overnight at 5° a yellow amorphous precipitate was obtained and isolated. The behaviour on the paper chromatogram was identical with that of the secondary component already described. The precipitated base was soluble in water but insoluble in diethyl-ether.

Chromatography of the Petroleum Layer.—Whereas mepacrine base was strongly adsorbed from petroleum on to alumina, the petroleum extract from liver was slowly developed into 3 green fluorescent bands and one leading blue fluorescent band. A separation into three or four bands was even more readily effected on a decalso column, which was preferable on account of the lower alkalinity of the sorbent. After preliminary trials on the small scale, 250 g. of decalso (sieved to 120–200 B.S.S.) was suspended in light petroleum, and poured into a glass tube to make a column of adsorbent, 2½ in. ×5 in. Into this was poured 750 ml. of petroleum fraction representing the extract from approximately 1,200 g. of mouse liver (1,100 mice). Development was continued with light petroleum (b.p. 60–80°). The fractions and their analyses are detailed in Tables II and III.

Determination of "Mepacrine Binding Power."—Two ml. of an *n*-hexane solution of the acid to be tested (containing about 0.05 m-equiv.) was placed in a 10 ml. B.14 glass-stoppered tube containing 2 ml. of aqueous 0.1M-phosphate buffer solution (*p*H 6). The contents of the tube were titrated with mepacrine base solution in *n*-hexane (1 mg./ml.) added dropwise with shaking after each addition. The end-point was reached at the first appearance of a yellow colour in the aqueous layer. The "mepacrine binding power" was defined as that amount of the sample which retained 10 mg. of mepacrine base in the petroleum layer.

Isolation of Acidic Phosphatide from Mouse Liver.—Livers (727 g.) from untreated mice were disintegrated in 1 l. of *iso*propanol. The treatment of the solids

TABLE II

CHROMATOGRAPHIC FRACTIONATION OF PETROLEUM EXTRACT FROM 1,200 G. OF LIVERS FROM TREATED MICE

Fraction No.	Treatment on Decalso Column and Subsequent Isolation
1	A brown band with purple fluorescence was eluted with light petroleum (b.p. 60–80°) and separated after extraction with 0·1 N-HCl into: (a) 83 mg. total solids from the acid layer and (b) 2·9 g. of yellow oil having a purple fluorescence after evaporation of the petroleum.
2	A yellow band with green fluorescence was eluted with light petroleum and extracted with *p*H 6 aqueous buffer: (a) 86 mg. hydrochloride was isolated from the aqueous layer. (b) 4·6 g. of oil was isolated from the petroleum layer. The oil was washed with methanol and chilled, and 0·202 g. of white crystals (m.p. 147° C.) remained.
3	A yellow band was eluted with 2 l. of petroleum ether and extracted with *p*H 6 aqueous buffer solution. (a) 106 mg. hydrochloride was isolated from the aqueous layer.* (b) 1·24 g. of oil remained after removing the petroleum.
4	A brown-yellow band was eluted with acetone, transferred to light petroleum and separated after extraction with *p*H 2 aqueous buffer into: (a) a yellow oil obtained from the aqueous layer after basification and extraction with chloroform. The oil did not yield a hydrochloride. (b) 0·86 g. of gum from the petroleum layer.
5	A fixed yellow band was excised from the top of the column and extracted with dilute hydrochloric acid: 26 mg. of hydrochloride was isolated from the aqueous acid extract.

* The hydrochloride was obtained from the aqueous solution after alkalinization, extraction into chloroform, transfer to dry diethyl-ether, and precipitation by passage of dry hydrogen chloride. The precipitated hydrochloride was washed with ether till free from acid.

obtained after spinning was repeated with two further 1 l. portions of *iso*propanol. The combined supernatant liquids were evaporated under reduced pressure and the concentrated aqueous-alcoholic layer extracted with light petroleum (b.p. 60–80°). The aqueous layer was rejected. To separate acidic phosphatide from neutral constituents the petroleum layer was extracted with 4N-sodium hydroxide. The extracted petroleum layer was rejected and the alkaline layer acidified whilst cooling, and extracted with two successive portions of *n*-hexane. The combined hexane layers were washed with water and evaporated to 25 ml.; 2.9 g. of total solids were in the hexane. The solids contained phosphorus and nitrogen but no sulphur, and had a mepacrine binding power of the same order (240 mg. binds 10 mg. of mepacrine) as that obtained from the treated liver (cf. Table II, fraction 2(b), after washing with acid).

Mepacrine Salt of Inositol Phosphatide.—Two g. of the inositol phosphatide fraction from ox-brain (Folch, 1942) was dissolved in 25 ml. of light petroleum (b.p. 40–60°), shaken with 25 ml. 2N-HCl and washed with water. After the addition of 200 mg. mepacrine base in 15 ml. of light petroleum, the colour changed to deep orange, indicating the formation of a salt. Water (40 ml.) was added and the petrol was removed *in vacuo*. Mepacrine phosphatide was obtained as a soft flocculated yellow precipitate. It was insoluble in water and acetone, sparingly soluble in ethanol and in cold propylene glycol, but soluble in diethyl-ether, in petroleum, and in hot propylene glycol.

RESULTS

TABLE III

IDENTIFICATION OF PRODUCTS ISOLATED

Description	Results of Infra-red Analysis, etc.*
A. *Water soluble metabolites isolated after counter-current extraction*	
Stages 1 and 2: 21 mg. ether insoluble solids	Broad band at 1,100 cm.$^{-1}$ indicated possible hydroxylated acridine.
144 mg. hydrochloride from ethereal solution.	Mepacrine hydrochloride.
Stages 3, 4 and 5: 480 mg. of hydrochloride isolated.	Mepacrine hydrochloride, containing from 5–10% of an unknown compound resembling that from stage 6.
Stage 6: 7 mg. ether insoluble base.	Possibly mepacrine metabolite having considerably modified side chain.
B. *Petroleum soluble metabolites isolated after passage down a "Decalso" column.*	
Fraction 1: 83 mg. total solids yielding 12 mg. after recrystallization from *n*-hexane.	8-chloro-3 : 5-dimethoxyacridine (m.p. 157°).†
Fraction 2: 144 mg. hydrochloride precipitated from ether.	Mepacrine hydrochloride.
4·5 g. of oil from the petroleum layer yielding 0·202 g. white crystals.	Crystals having m.p. 147° and free from nitrogen, sulphur and phosphorus.
Fraction 3: 106 mg. hydrochloride precipitated from ether.	Mepacrine hydrochloride.
Fraction 4:	Not identified.
Fraction 5: 20 mg. hydrochloride from top of column.	Mepacrine hydrochloride.

* The conclusions on the i.r. spectra are based on comparison with the spectra of mepacrine analogues bearing modified side chains. Several of the latter are formally analogous to metabolites identified by Titus *et al.* (1948) in metabolic studies of 4-aminoquinoline antimalarials.

† 8-chloro-3 : 5-dimethoxyacridine (m.p. and mmp. 157°) arose by interaction of mepacrine with methanol during the course of isolation, even although the conditions were very mild. *iso*-Propanol was used later as a less reactive alternative. 8-chloro-3 : 5-dimethoxyacridine is a weak base, is not extracted from petroleum by aqueous buffer of *p*H higher than 2, and is distinguished by a brilliant violet fluorescence even in non-polar solvents.

Identification of Acid Phosphatides as Mepacrine Binding Agents

It was noted above that whereas mepacrine base was strongly adsorbed from petroleum solution on to decalso, the crude petroleum extract from the livers of treated mice was developed into several bands or fractions. Also, although mepacrine could not be extracted from the crude product before column treatment either with *p*H 6 buffer or with 0.1N-HCl, yet mepacrine was readily extracted by *p*H 6 buffer from the main petroleum fractions 2, 3, and 5, eluted from the column. This behaviour could be explained by postulating a petrol-soluble organic acid salt of mepacrine base which, although

initially stable on treatment with dilute hydrochloric acid, exchanged mepacrine cation for sodium on passage down the decalso column (decalso is, in fact, an ion-exchange medium), so that the free mepacrine base subsequently eluted would be readily extracted with *p*H 6 buffer solution. The hypothesis was supported by the following observations: (i) when 340 mg. of the oily residue from the fraction 2b (Table II, the main acridine fraction) was dissolved in hexane, shaken with 1N-HCl, then washed with water, it had regained the ability to retain mepacrine base (10 mg.) against the extractive action of aqueous 0.1N-HCl solution. The object of acid washing the oil is to replace the metal cation picked up from the column by hydrogen. (ii) Potentiometric titration of 2b after acid treatment was carried out using a micro-technique. The titration curve was that of a weak acid (15.5 g.≡1 m-equiv. NaOH). The amount of oil which retained 1 m-equiv. of mepacrine base (m.w. 400) against extraction with 0.1N-HCl was of a similar order (13.6 g.).

Since a number of higher fatty acids (tested both as individuals and as mixtures obtained after saponification of linseed and castor oils), yeast nucleic acid and tauroglycocholic acid had no mepacrine binding power, it was decided to look for an appropriate fraction in the livers of undosed mice. After isolation as described above the product had a mepacrine binding power comparable with the oil isolated from the chromatographic column fraction 2b, and it contained phosphorus and nitrogen. The presence of phosphorus indicated that acidic phospholipids might be responsible for the effect, and in fact, when soya-bean lecithin in petroleum solution was shaken with 2N-HCl solution, then washed acid-free, the product had marked mepacrine binding power. As there are a number of phospholipids in this lecithin, the affinity of the acid phosphatides for mepacrine was more particularly investigated by preparing the phosphatidyl serine and inositol phosphatide fractions from ox-brain (Folch, 1942). After shaking with 2N-HCl and washing free from acid, approximately 80 mg. of these fractions in petroleum bound 10 mg. of mepacrine base against extraction with 0.1N-HCl. The addition of the phospholipids to mepacrine base in petroleum produced a colour change from very pale yellow to orange, which suggested salt formation.

In order to obtain an indication of the acid radical in the phosphatide obtained from the control mouse liver, part of the hexane solution was refluxed for three hours with 5N-HCl. The lower layer was removed, washed with hexane and then evaporated to dryness. Water was added to the residue, which was again later evaporated to dryness to remove hydrochloric acid. Paper strip chromatography of the residue developed with butanol-propanol-water (47 : 28 : 25 parts by volume) resulted in a spot reacting with ninhydrin and having an R_F value similar to that of a serine control.

Discussion

It has been shown above that when mice are given mepacrine hydrochloride (10 mg./mouse) by mouth, and a suitable period (48 hours) allowed for metabolism, approximately 94% of the total acridine in the livers was recovered as mepacrine. The remaining 6% was presumed to be metabolites (Table IV, c, d, and e ; b is an artefact). If these had contained a highly active constituent an antiviral effect should have been demonstrable from a small parenteral dose. However, at a level of 0.1 mg. b.i.d. for 5 days no activity was apparent, whereas mepacrine at 0.5 mg. b.i.d. for 5 days showed marked activity. It would appear, then, that the metabolites isolated do not contribute materially to the total antiviral effect.

A considerable proportion (26–38% according to the preparation) of the mepacrine was isolated in the form of acid phosphatide salts. These are soluble in fat solvents, insoluble in water, and not

Table IV

BIOLOGICAL TESTS

Mice (18–20 g.) were given drug by mouth and then infected with Eastern equine encephalomyelitis. The numerals in parentheses indicate the mean period of survival in days.

Ref.	Compound	Dose (g.) and Route	Deaths/20
a	Control		16 (4·7)
	Mepacrine	10 oral once	0
		0·5 i.p. once	18 (6·0)
		0·5 i.p. b.i.d. 5 days	8 (6·1)
	Mepacrine phosphatide	0·5 i.v. once	17 (5·5)
		0·5 i.v. b.i.d. 5 days	18 (4·7)
		0·5 i.p. once	18 (6·8)
		0·5 i.p. b.i.d. 5 days	12 (6·3)
b	Control		16 (5·6)
	Mepacrine	10 oral once	3 (9·6)
	8-Chloro-3 : 5-dimethoxy-acridine	10 oral once	14 (5·7)
		10 oral in oil	16 (6·2)
		5 i.v.	18 (5·5)
c	Control		9 (5·0)
	Mepacrine	0·1 i.p. b.i.d. 5 days	7 (6·6)
	Ether insoluble solids (Table IIIA stages 1 and 2)	0·1 i.p. b.i.d. 5 days	9 (4·9)
d	Ether insoluble base (Table IIIA stage 6)	0·1 i.p. b.i.d. 5 days	10 (5·4)
e	Control		9 (5·7)
	Mepacrine	0·1 i.p. b.i.d. 5 days	10 (5·6)
	Secondary component from stage 3–5, Table IIIA	0·1 i.p. b.i.d. 5 days	10 (5·7)

readily decomposed by dilute hydrochloric acid. It is quite probable that the mepacrine phosphatides are formed within the tissue, since, in preliminary work, acid stable, solvent soluble, mepacrine derivatives were not found after homogenizing mouse liver with mepacrine. These salts presumably account for the persistent staining noted in liver and other organs. This leads naturally to the idea of a depot preparation, but the injection of mepacrine diphosphoinositide (0.5 mg., i.p., b.i.d. 5 days) did not lead to promising results.

Summary

1. Mice were given mepacrine hydrochloride by mouth. After 48 hours, the animals were killed and the livers examined for metabolites. Of the acridine extracted, 94% was mepacrine ; the remainder was separated into three distinct fractions by countercurrent distribution methods. The fractions contained metabolites having infra-red spectra comparable to those of model compounds in which the side-chain had been modified. The metabolites had no significant biological activity against Eastern equine encephalomyelitis in the mouse (after i.p. injection).

2. From 26 to 38% of the mepacrine isolated was in the form of phosphatide salts, which are very soluble in fat solvents and are not decomposed by dilute hydrochloric acid. The persistence of mepacrine in tissue is probably due in part to the formation of these salts with the cell lipids. Yet it is not possible to attribute depot effects to these salts, since the mepacrine salt of an acidic phosphatide prepared from ox-brain was not superior to mepacrine hydrochloride as an antiviral drug in the mouse.

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