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Distribution and excretion of N-[^{14}C]-methyl labelled *m*-hydroxybenzyltrimethylammonium ions in mice

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Summary

1. Radioactivity in the plasma, livers, and brains of mice was measured at intervals of 10 min to 24 h after the intraperitoneal injection of ^{14}C labelled *m*-methoxy- and *m*-hydroxy- benzyltrimethylammonium ions.

2. Well over 10% of the dose of either of these quaternary compounds was found in the liver and plasma within 10 min of the injection, but they were rapidly excreted or metabolized, having a half life of between 1 and 2 hours.

3. Small amounts of radioactivity were detectable in brain, and were not due to contamination of brain samples by blood, after doses of 14–16 μmol/kg (approximately 4 mg iodide/kg) and upwards. After doses of 4–6 μmol/kg (approximately 1 mg iodide/kg), however, radioactivity in the brain was barely detectable.

4. Any behavioural effects reported to occur in mice treated with these lower doses of *m*-hydroxybenzyltrimethylammonium, which include improved performance in a test of avoidance learning, are unlikely to be due to an action within the central nervous system. Learning may be more influenced by the peripheral effects of drugs than has hitherto been appreciated.

Introduction

m-Hydroxybenzyltrimethylammonium ions have considerable nicotine-like activity on the frog rectus preparation (Barlow & Thompson, 1969) and resemble nicotine in apparently facilitating the acquisition by mice of a conditioned avoidance response (Barlow, Oliverio, Satta & Thompson, 1970). Several other quaternary ammonium compounds were reported to have similar properties and it was suggested that ' the possibility that they can penetrate into the central nervous system should not be ruled out ', even though they are permanent cations. It is possible to see why quaternary compounds can impair the acquisition of a conditioned avoidance response by a peripheral action but more difficult to see how they may improve it without acting centrally. This paper describes an attempt to assess the ability of *m*-hydroxybenzyltrimethylammonium ions to penetrate into the central nervous system by measuring the radioactivity in the brain, liver and plasma of mice following injection of N-$^{14}CH_3$ labelled *m*-hydroxybenzyltrimethylammonium ions by the same route (intraperitoneal) as was used in the tests on conditioned avoidance responses. The *m*-methoxy compound, from which the *m*-hydroxy compound was prepared, was also studied for comparison. It was inactive in the conditioned avoidance tests and so might be regarded as a control.

Methods

N-[^{14}C]H_3-labelled *m*-methoxybenzyltrimethylammonium iodide was supplied by the Radiochemical Centre, from the methylation of *m*-methoxybenzyldimethylamine, b.p. 108°/10 mm, N_D^{25} 1·5116, with [^{14}C]H_3I. The material had m.p. 145–7° (Barlow & Thompson, 1969, recorded m.p. 143–144°) and a specific activity of 4·0 mCi/mmol.

This material (139 mg) was refluxed under nitrogen with concentrated (48%) aqueous hydrobromic acid (3·3 ml) for 18 h and then neutralized with aqueous sodium bicarbonate (molar). The solution was concentrated under reduced pressure and the residue extracted with ethanol and filtered from insoluble sodium bromide. The extract was concentrated, re-extracted with ethanol and refiltered twice more (making 3 times in all) and finally dissolved in 1·5 ml deionized water. This solution contains traces of the methoxy compound which was detected by its ultraviolet absorption spectrum. The methoxy compound has λ_{max} 276 nm, $\epsilon_{molar}=1{\cdot}88\times10^3$ and this is unaffected by changes in pH. The demethylated compound also has a maximum at 300 nm, due to the phenoxide ion, and at pH 9·2 the combined effect produces a double-humped curve with a maximum at 300 nm ($\epsilon_{molar}=1{\cdot}78\times10^3$) and a shoulder at 287 nm ($\epsilon_{molar}=1{\cdot}58\times10^3$). The presence of undemethylated material was apparent when the concentrated solution was diluted with buffer, pH 9·2, and the shoulder was more prominent than that observed with pure *m*-hydroxybenzyltrimethylammonium iodide.

The mixture was subjected to electrophoresis on paper in buffer at pH 11·4–12·0, obtained by adding 20 ml N NaOH to 500 ml buffer pH 9·2 prepared from 'Soloid' buffer tablets (Burroughs Wellcome). At this pH the phenolic compound is a zwitterion and was detected along the line at which it was applied, whereas the methoxy-compound moves to the cathode. The paper was scanned, cut up and eluted with water and boiling methanol, the eluates were concentrated and the residue taken up in deionized water (10 ml). When 0·1 ml of this solution was diluted to 10 ml with buffer, pH 9·2, the ultraviolet absorption spectrum in the region 260–300 nm was identical with that of pure *m*-hydroxybenzyltrimethylammonium iodide and indicated that the concentration was $2{\cdot}58\times10^{-4}$M so that of the parent solution was $2{\cdot}58\times10^{-2}$M. The amount of *m*-hydroxy compound obtained was approximately 80 mg (calculated as iodide) and another 5 mg was obtained by further elution of the paper with boiling methanol. The anion present in the solution is, in fact, determined by the composition of the buffer (borate) but weights of doses quoted in this paper have been calculated from the ionic concentrations and expressed in terms of the iodide for comparison with previous work and with the results obtained with the methoxy compound. The specific activity was 3·9 mCi/mmol and it can be seen that, as expected, the O-demethylation has not been accompanied by any significant N-demethylation. Benzyl quaternary ammonium salts are particularly stable chemically because they cannot undergo a Hofmann degradation (in contrast phenethylquaternary ammonium salts may be converted to styrenes in alkaline conditions).

The mice used were males, strain C 57, weighing 20–30 g. Drug solutions for injection were made up in 0·9% sodium chloride solution and administered intraperitoneally in a volume not exceeding 0·1 ml. Labelled methoxy compound was tested in doses of 4·3 and 14·3 μmol/kg (1·3 and 4·4 mg iodide/kg); labelled hydroxy compound was tested in doses of 5·5, 15·5, and 57·4 μmol/kg (equivalent to 1·6, 4·5 and 16·8 mg iodide/kg). None of these doses appeared to produce any

obvious effects in the mice. Control animals did not receive any injection. Groups of two or four mice were killed by the intraperitoneal injection of sodium pentobarbitone (0·05 ml of a solution 60 mg/ml) 10 min and 30 min and, in some experiments, 2 h, 4 h and 24 h, after they had been given the labelled material. Blood was removed from the venous sinus behind the eye with a heparinized capillary tube, and from the heart, and placed in a polyethylene centrifuge tube (capacity 1 ml) containing 4 units of heparin/ml blood collected. The plasma, which always contained some haemoglobin from haemolysed red cells, was separated by centrifuging in a Zeiss Eppendorf Zentrifuge 3200 for one minute.

The whole brain was removed, cut transversely, and washed in 0·1 M sodium acetate buffer, pH 5·0, to remove traces of blood and cerebrospinal fluid. It was then homogenized in 5 volumes of 0·1 M sodium acetate buffer, pH 5·0, with a Teflon homogenizer.

The right median and left lateral lobes of the liver were removed (care was taken not to include the gall bladder), washed, and homogenized in 5 volumes of 0·1 M sodium acetate buffer, pH 5·0, in the same way as the brain.

The liver and brain homogenates were frozen for 1–2 h to assist aggregation of protein and then centrifuged for 15 min at 3,000 r.p.m. and room temperature. They were stored at 4° C overnight, centrifuged for a further 15 min at 3,500 r.p.m. and 4° C, and the supernatant was then clear and its volume was recorded.

The radioactivity of portions (0·1 ml) of the plasma, and of the brain and liver supernatants was counted with a Beckman scintillation counter (LS-100) and a dioxan scintillant containing 0·7% 2,5-diphenyloxazole (PPO) and 0·03% 1,4-di(2-(5-phenyloxazolyl)) benzene (POPOP). All values were corrected for quenching by using an external standard. The samples were left overnight in the counter to equilibrate before they were counted for a 20 min period at least 3 times. The activity was expressed as d.p.m./ml of plasma or of tissue supernatant. If it is assumed that these small quaternary ions do not bind significantly to tissue proteins, the radioactive material should be extracted into the supernatant and the measurements with this should make it possible to calculate the radioactivity as d.p.m./g of liver or brain.

In some of the brain extracts the concentration of haemoglobin was measured by the cyanmethaemoglobin method (Varley, 1969) to see to what extent they were contaminated with blood.

Results

Table 1 shows the radioactivity in plasma and brain 10 min and 30 min after the injection of the labelled compounds. The activities are expressed as counts recorded in excess of the non-specific counts produced by the controls (possibly by chemiluminescence).

The mean of the estimates of the concentration of blood in the brain extracts was 0·94 μl/g brain, with the range 0·48–1·7 μl/g brain. This corresponds to about 0·5 μl plasma/g brain and the contribution that this makes to the radioactivity of the samples is small. With a plasma level of 20,000 d.p.m./ml it would only be 10 d.p.m.

In the dose range 4–6 μmol/kg, which is comparable with that used in the behavioural studies, there is no significance in the amount of radioactivity in the brain

TABLE 1. *Uptake of* [^{14}C]-m-*methoxybenzyltrimethylammonium ions and* [^{14}C]-m-*hydroxybenzyltrimethylammonium ions into mouse plasma and brain after intraperitoneal injection*

Dose range (μmol/kg)	Min after injection (*n*=no. of mice)	[^{14}C]-*m*-methoxybenzyltrimethylammonium ion		[^{14}C]-*m*-hydroxybenzyltrimethylammonium ion	
		Plasma	Brain	Plasma	Brain
4–6	10 (*n*=2)	25,760±3,900	420± 260	20,790± 2,500	200±200
	30 (*n*=2)	not done	970±1,370	30,940± 500	0
14–16	10 (*n*=4)	43,400±2,750	880± 480	54,860±10,070	1,130±640
	30 (*n*=4)	27,510±7,180	850± 200	38,930±16,260	1,240±980
57·4	10 (*n*=2)			211,340± 9,300	3,250±500
	30 (*n*=2)			148,600±19,800	2,020±520
Non-specific counts in untreated animals	(*n*=6)	910± 190	2,360± 140	1,080± 350	2,520±300

Figures show the mean d.p.m./ml (plasma) and d.p.m./gram (brain) ± S.D., in excess of the non-specific counts from the untreated animals (which are shown at the foot of the table). The doses in the range 4–6 μmol/kg were 4·3 and 5·5 μmol of the methoxy and hydroxy compounds respectively, equivalent to 1·3 and 1·6 mg iodide/kg, and comparable with those used in the behavioural studies (Barlow, Oliverio, Satta & Thompson, 1970). In the range 14–16 μmol they were 14·3 and 15·5 μmol/kg respectively. Values for the brain extracts are only slightly affected by contamination with blood, see text.

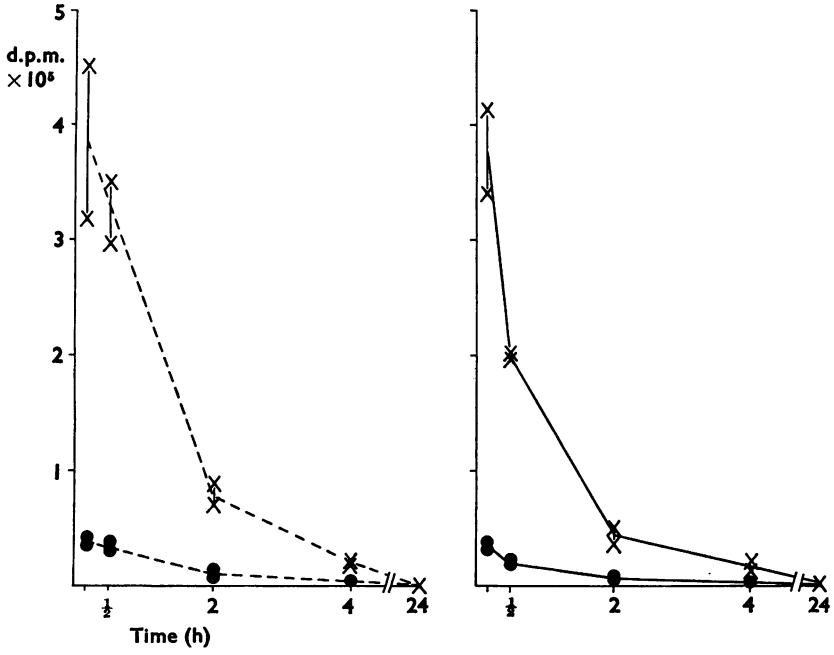

FIG. 1. Absorption of [^{14}C]-*m*-methoxybenzyltrimethylammonium ions (dashed line) and [^{14}C]-*m*-hydroxybenzyltrimethylammonium ions (full line) into the plasma (●) and liver (×) of mice after intraperitoneal injection of doses in the range 14–16 μmol/kg (approximately 4 mg iodide/kg). Ordinate, disintegrations per minute; abscissa, time in hours after intraperitoneal injection.

The total amounts of radioactivity administered were, for the *m*-methoxy compound, equivalent to $37{\cdot}84\times10^5$ d.p.m./30 g mouse and for the *m*-hydroxy compound equivalent to $29{\cdot}92\times10^5$ d.p.m./22 g mouse (the two experiments were performed on different batches of mice). Each point represents the estimate of the d.p.m. corresponding to the whole liver or whole body plasma calculated from the mean count (see **Methods**) of a sample from one mouse. Values for brain were too small to be indicated.

after injection of the labelled material. In the dose range 14–16 μmol/kg, which is more than twice the dose of the hydroxy compound which was reported to improve avoidance learning, the levels of radioactivity were still only 50% in excess of the control levels and certainly not significantly different from those following the administration of the methoxy compound, which was inactive in the behavioural tests. With the highest dose, which corresponds to nearly 17 mg/kg, the radioactivity in the brain 10 min after injection was about 130% in excess of the controls. This would be equivalent to a concentration of about 4×10^{-4} μmol/g or roughly 4×10^{-7}M, which is about one-fiftieth of the concentration of the compound which produced some degree of contracture of the frog rectus preparation.

In Fig. 1 the radioactivity in the whole liver and total body plasma are plotted against time after intraperitoneal injections of doses of 15·5 μmol/kg of *m*-hydroxybenzyltrimethylammonium and 14·3 μmol/kg of the *m*-methoxy compound. Values for the plasma were calculated assuming that the total body blood in the mouse is 5·77% of the body weight (Kleiber, 1961). Values for the brain were too low to be included. The absorption into the plasma and the concentrations in the liver are remarkably similar for the two drugs; 10 min after the injection 1% of the injected dose of the methoxy derivative and 1·2% of that of the hydroxy compound were present in the total plasma and 10% of the methoxy compound and 13% of the hydroxy compound were found in the liver. Clearance of both drugs from plasma and liver were similarly rapid, only 2% of the injected dose being detectable in the plasma and liver together after 4 hours.

Discussion

After intraperitoneal injection it is clear that appreciable quantities, well over 10%, of these quaternary ions are absorbed systemically and are also excreted or metabolized rapidly, the substances having a half life in mice of between 1 and 2 hours. There does not appear, however, to be any significant penetration into the brain. It seems therefore that any effects which *m*-hydroxybenzyltrimethylammonium might have in improving avoidance learning must be ascribed to peripheral actions. Though an action such as some sort of blockade of the neuromuscular junction could impair performance, it is more difficult to see how performance could be improved by peripheral effects but cerebral vascular changes could be involved. Dr. Armitage very kindly tested this compound and found that it did not produce the ear twitches characteristic of centrally acting nicotine-like compounds (Armitage, Milton & Morrison, 1966), when injected intravenously. In contrast the *m*-hydroxyphenylpropyltrimethylammonium homologue did do so. It seems possible therefore that some quaternary ions, such as this latter compound might penetrate into the central nervous system but, unless the penetration of these compounds into the brain is remarkably different in the strain of mice which we have used from the DBA/2J strain used in the behavioural work, our failure to find significant amount of labelled material in the brain suggests that the results obtained by Barlow *et al.* (1970) in an avoidance learning test might have been influenced by the peripheral effects of a drug, a concept that deserves further investigation.

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