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HYDROLYSIS OF NEOSTIGMINE BY PLASMA CHOLINESTERASE

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Incubation of neostigmine with normal human plasma *in vitro* results in the formation of two quaternary nitrogen compounds, one of which has been identified as *m*-hydroxyphenyltrimethylammonium. This hydrolysis is prevented by prior addition of dyflos to plasma in concentrations sufficient to inhibit plasma cholinesterase activity. The significance of these findings is discussed in relation to the occurrence of the same metabolic products in the urine of patients with myasthenia gravis treated with oral neostigmine. No equivalent findings are available for normal subjects since it was not considered justifiable to treat them with neostigmine.

In the course of investigating the metabolism and excretion of neostigmine in patients with myasthenia gravis, we obtained evidence that two metabolites were excreted in the urine, both of which were quaternary nitrogen compounds. One of them was subsequently identified as *m*-hydroxyphenyltrimethylammonium (Scott, Nowell & Wilson, 1962). The excretion of these metabolites seems to depend upon the route of administration of neostigmine, because after intramuscular injection only unchanged neostigmine was detected in the urine, whereas after oral administration no neostigmine was excreted but only the two metabolic products referred to above.

These findings led us to investigate the possible factors involved in the metabolism of this drug. Since neostigmine is an anticholinesterase drug and its hydrolytic product, *m*-hydroxyphenyltrimethylammonium, was detected in urine, we considered it appropriate to determine whether cholinesterase was involved in this reaction. The effects of incubating neostigmine with plasma were investigated to determine whether this would result in the formation of *m*-hydroxyphenyltrimethylammonium or any other metabolic products of neostigmine. Similar studies were also made using plasma to which dyflos had previously been added in concentrations sufficient to inhibit its cholinesterase activity.

METHODS

Incubation of neostigmine with normal human plasma. Fresh human plasma was obtained from normal subjects and in a few experiments reconstituted dried human plasma was used. Coagulation was prevented by adding potassium oxalate 2 mg/ml. Neostigmine was added to 5 ml. plasma to produce a concentration of 100 μg/ml. and incubated in glass tubes plugged with cotton-wool in a water bath at 37.5° C for periods of 4 to 24 hr. The samples of plasma were then extracted by the Sephadex method.

Sephadex treatment of plasma containing neostigmine. A column 2 cm by 26 cm was prepared by adding water to 16 g dry weight of Sephadex G-25 resin (Savory & Moore, London,

for Pharmacia, Sweden). 5 ml. plasma containing neostigmine was run through the column, which was then eluted with distilled water and two fractions were collected. The first fraction (50 ml.) which contained the plasma proteins was discarded; the second fraction (30 ml.) was evaporated to dryness on a boiling water bath. The resultant residue, which contained salts and quaternary nitrogen compounds, was extracted with 1 ml. ethanol; 0.5 ml. of the supernatant fluid was used for chromatographic investigation.

Paper chromatography. The paper chromatographic methods, using the solvent system, 8 butanol:2 ethanol:3 water:0.25 acetic acid, were those previously described (Nowell, Scott & Wilson, 1962).

Dyflos-treated human plasma. Dyflos (DFP) was chosen as a convenient inhibitor of pseudocholinesterase activity, and consistent results were obtained when samples of human plasma were incubated at 37.5° C for 30 min with concentrations of 1×10^{-7} M to 5×10^{-7} M dyflos.

To ensure that all the experiments were conducted with samples of plasma in which the pseudocholinesterase was completely inhibited, it was necessary to select a suitable method for measuring the cholinesterase activity in plasma. The method described by Kalow & Lindsay (1955) was found to be simple and convenient. This depends on estimating the rate of hydrolysis of benzoylcholine when added to plasma diluted with phosphate buffer at *p*H 7.4. Benzoylcholine was estimated spectrophotometrically at 240 mμ. The change in optical density was recorded for 20 min and was a measure of the rate of hydrolysis of benzoylcholine and therefore of the cholinesterase activity of plasma.

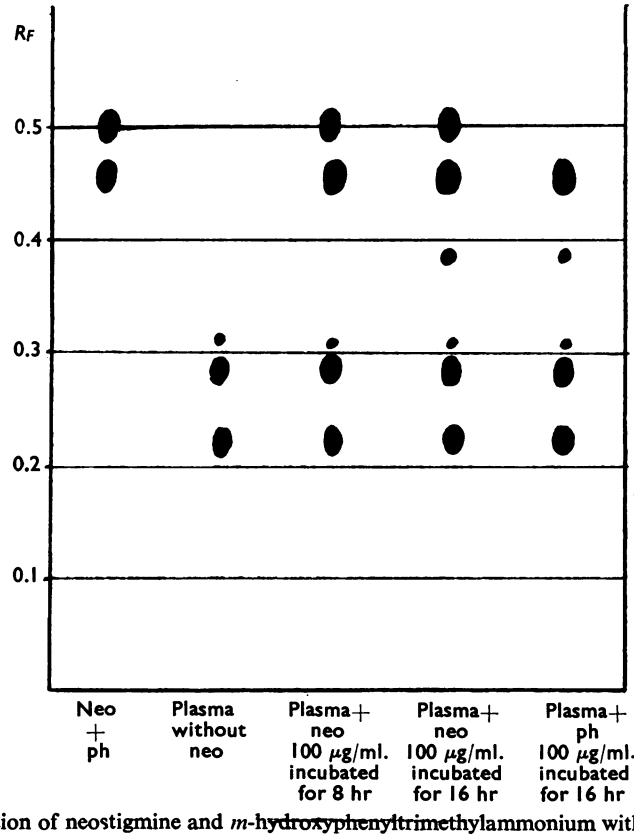

Fig. 1. Incubation of neostigmine and *m*-hydroxyphenyltrimethylammonium with plasma. Neo= neostigmine bromide or methylsulphate; ph=*m*-hydroxyphenyltrimethylammonium bromide.

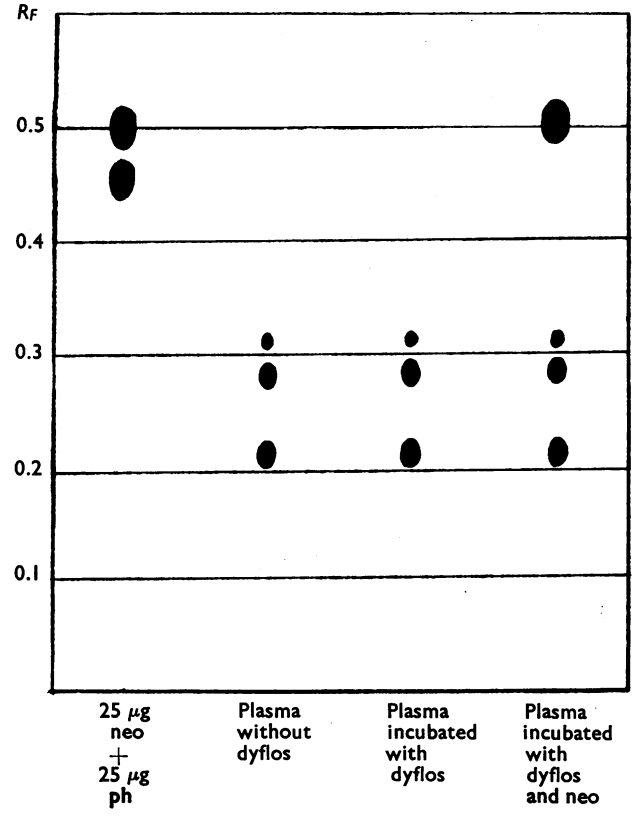

Fig. 2. Incubation of plasma with dyflos and neostigmine. Neo=neostigmine bromide or methylsulphate; ph=*m*-hydroxyphenyltrimethylammonium bromide.

RESULTS

Incubation of neostigmine with plasma. The ethanol extract of the residue obtained from the eluate of the Sephadex treatment of each sample of plasma incubated with neostigmine was subjected to paper chromatography, the results of which are illustrated in Fig. 1.

Here it will be seen that the spots detected are related to the duration of incubation of neostigmine with plasma. Thus, after 8 hr incubation, two spots were detected, one at R_F 0.49 to 0.51, which was regarded as neostigmine; a second spot in close proximity at R_F 0.44 to 0.46 gave a characteristic blue colour with iodoplatinate reagent and was considered to be *m*-hydroxyphenyltrimethylammonium.

By contrast, after 16 hr incubation, in addition to the two spots already mentioned, a further spot was detected at R_F 0.37 to 0.39. This was tentatively assumed to be a derivative of *m*-hydroxyphenyltrimethylammonium, and further evidence in support of this conclusion was provided by the results of incubating plasma for 16 hr with *m*-hydroxyphenyltrimethylammonium bromide, as shown in the last column of Fig. 1.

In each chromatogram of incubated plasma three spots were detected at R_F values lower than 0.35. Identification of these was not considered immediately relevant to the investigation, since they were also obtained with samples of plasma to which no neostigmine or *m*-hydroxyphenyltrimethylammonium bromide had been added, and occurred whether or not the plasma was incubated.

Incubation of neostigmine with normal human plasma in the presence of dyflos. In these experiments six samples of normal human plasma were used. When the concentration of dyflos which just produced complete inhibition of cholinesterase activity had been determined for each sample, aliquots were then incubated with neostigmine for 4 to 24 hr, extracted by the Sephadex method previously described, and chromatographed. As will be seen from Fig. 2, which represents a typical result, no spots were detected other than neostigmine and the three spots referred to above; the hydrolysis of neostigmine to *m*-hydroxyphenyltrimethylammonium was thus prevented by dyflos.

DISCUSSION

These results have shown that when neostigmine is incubated with human plasma it undergoes transformation with the formation of two products one of which has been identified as its phenolic derivative, *m*-hydroxyphenyltrimethylammonium. Since this change does not occur when dyflos is present in concentrations sufficient to inhibit completely the pseudocholinesterase of plasma it is reasonable to assume that pseudocholinesterase is intimately involved in the hydrolysis of neostigmine to *m*-hydroxyphenyltrimethylammonium.

Scott, Nowell & Wilson (1962) have reported that *m*-hydroxyphenyltrimethylammonium and an unknown metabolic product of neostigmine are present in the urine of myasthenic patients treated orally with neostigmine. There is therefore some resemblance between the effects of incubating neostigmine *in vitro* with plasma and the metabolic changes which occur when the drug is administered by mouth.

Within the limits of the experimental conditions used it seems reasonable to conclude that neostigmine is first hydrolysed to its phenolic derivative, *m*-hydroxyphenyltrimethylammonium, and that a further change, probably of the phenolic derivative, results in the formation of another substance of R_F value approximately 0.38. The fact that these changes occurred only after incubation for at least 8 hr implies that the reactions involved are slow, and this would seem to be inconsistent with the fact that the *m*-hydroxy derivative was detected in urine 4 hr after an oral dose of neostigmine (Scott, Nowell & Wilson, 1962). It is important to appreciate, however, that whereas in patients the concentration of neostigmine or its metabolites in plasma is not likely to exceed 1 μg/ml., in the *in vitro* work the concentrations used were 100 times greater, and this might alter the reaction rates. The fact that *m*-hydroxyphenyltrimethylammonium has some anticholinesterase activity at high concentrations (Wescoe, Riker & Beach, 1950) may also contribute to the slowness of the reaction *in vitro*.

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