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METABOLISM OF [^{14}C]-NEOSTIGMINE IN THE RAT

BY

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When neostigmine is administered orally to patients with myasthenia gravis two metabolites are excreted in the urine one of which has been identified as *m*-hydroxyphenyltrimethylammonium (Scott, Nowell & Wilson, 1962). It was later reported by these investigators that after prolonged *in vitro* incubation of neostigmine with human plasma two metabolites were identified; these were not present when plasma had been previously incubated with dyflos (Nowell, Scott & Wilson, 1962b). The fact that no metabolites could be detected in the urine of myasthenic patients who had been given neostigmine by intramuscular injection (Nowell, Scott & Wilson, 1962a) and that the hydrolysis of neostigmine *in vitro* was very slow suggested that some tissue other than plasma might be concerned in the metabolism of the drug.

Recent work in this laboratory has shown that in the rat, after intramuscular injection of [^{14}C]-neostigmine, radioactivity is rapidly concentrated in the liver and slowly declines to negligible amounts within 24 hr (Roberts, Thomas & Wilson, 1965). This evidence strongly suggests that the liver is an important organ in the metabolism of neostigmine and is supported by the results described in this paper which is concerned with the estimation of unchanged drug and metabolite in the urine and liver of the rat after intramuscular injection of [^{14}C]-neostigmine.

METHODS

[^{14}C]-Neostigmine iodide, supplied by the Radiochemical Centre, Amersham, had a specific activity of 15 μc/mg and was used to examine its excretion in the urine and distribution in the liver of the rat. Male rats weighing 150 to 200 g were hydrated and injected intramuscularly into the hind-limb with a standard dose of 25 μg of [^{14}C]-neostigmine in 0.1 ml. of water; urine was collected and the liver was extracted and estimated for total radioactivity by the methods previously described (Roberts *et al.*, 1965). In the later experiments with SKF 525-A (2-diethylaminoethyl-3,3-diphenylpropylacetate) the method for extraction from liver was modified by evaporating the extract to 1 ml. instead of 10 ml. and then adjusting the volume to 10 ml. with absolute ethanol. After settling, the supernatant fluid was used to estimate metabolite and neostigmine.

Estimation of neostigmine and metabolite. Although the metabolite has not been separated and unequivocally identified as *m*-hydroxyphenyltrimethylammonium, there is fairly strong evidence from its R_F on paper chromatography and its mobility on paper electrophoresis to support this assumption (Scott *et al.*, 1962). To separate the metabolite from neostigmine in urine and liver extracts a paper electrophoresis method was developed.

Specimens of urine were applied as a spot, and extracts of liver as a line, to Whatman 541 paper (33 × 4 cm) at a point 7.5 cm from one end and dried by a current of hot air (Nowell *et al.*, 1962a) or with an infrared lamp placed underneath the paper. The liver extracts were applied to the paper through a fine polyethylene

cannula attached to a syringe driven by a Palmer continuous slow perfusion apparatus. The excursions of the tip of the cannula along the line of origin were controlled by a reversible electric motor. The papers were moistened with borate buffer (0.1 M, *p*H 9.2) using a Pasteur pipette and, with the origins nearest the anode compartment, they were run at 200 V and 0.25 mA/cm width for 2.5 hr. They were dried horizontally in an air oven at 100° C for 10 min and cut transversely into serially numbered strips 1 cm wide. Each strip was rolled in the form of a cylinder, placed in the scintillation fluid (Kinard, 1957) and counted. In developing this method we found that radioactivity in urine separated into two distinct peaks; control experiments showed that the counts in strips 10 to 13 were a measure of neostigmine and those in strips 5 to 9 of the metabolite (Fig. 1). The collective counts for each of the two zones were expressed as a percentage of the total count of the sample. From this it was possible to derive the proportions of the metabolite and unchanged drug which were excreted in the urine or contained in the liver.

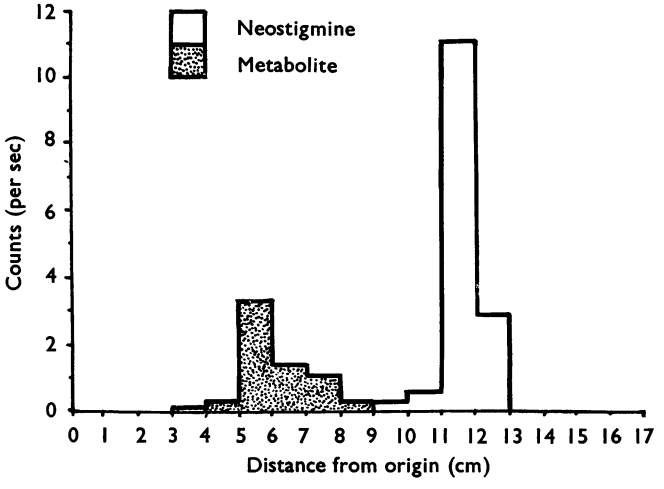

Fig. 1. Separation of metabolite from neostigmine in rat urine by paper electrophoresis. The shaded area (5 to 9 cm) represents radioactivity due to metabolite and the unshaded area (10 to 13 cm) that due to neostigmine.

Drugs. The basic cyanine dye 1'-ethyl-3,6-dimethyl-2-phenyl-4-pyrimido-2'-cyanine chloride (Cyanine 863) was used as a freshly prepared solution in 0.9% saline. SKF 525-A was administered as a freshly prepared solution of 3.75 mg in 0.5 ml. of water.

RESULTS

Urine. The excretion of neostigmine and the metabolite in urine at different time intervals after intramuscular injection of [^{14}C]-neostigmine (25 μg) is shown in Fig. 2. The results are expressed as a percentage of the dose and are derived as a mean of observations on four to seven individual rats. Fig. 2 also includes the results of estimating total radioactivity. During the first 30 min there is a rapid excretion of radioactivity which can be largely attributed to neostigmine; the elimination of unchanged drug virtually ceases at 1 hr. The slow increase in output of the metabolite is also clearly evident but it is noteworthy that even within 15 min of injection the metabolite can be detected. After 1 hr the curve for the output of the metabolite closely parallels the output of total radioactivity.

We have previously shown that the excretion of radioactivity in the urine of the rat and hen after intramuscular injection of [^{14}C]-neostigmine can be inhibited by prior administra-

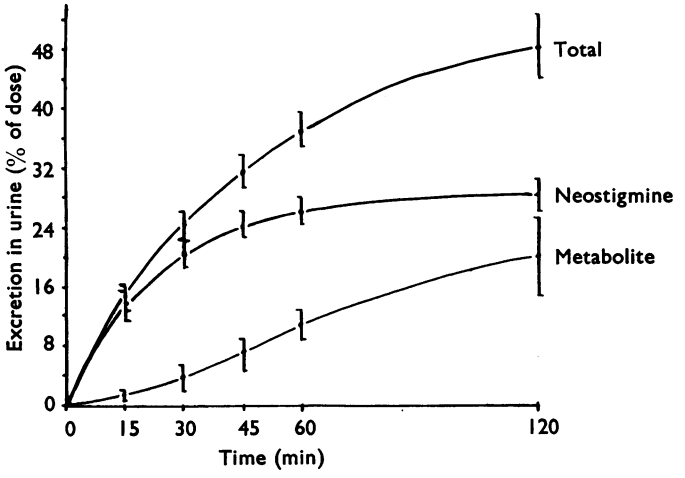

Fig. 2. Excretion of neostigmine, of metabolite and of total radioactivity in the urine of the rat after intramuscular injection of 25 μg of [^{14}C]-neostigmine. Each point is the mean of four to seven experiments. The standard deviations are represented by the vertical lines.

tion of Cyanine 863 (Roberts, Thomas & Wilson, 1963; Roberts *et al.*, 1965). The inhibitory effect of Cyanine 863 on the excretion of neostigmine and the metabolite was therefore studied in four rats by injecting it intramuscularly 1 hr before the intramuscular injection of neostigmine. The molar ratio of Cyanine 863 to neostigmine was 2 : 1 in two experiments and 4 : 1 in the others. The results are shown in Table 1. The excretion of total radioactivity is inhibited by Cyanine 863; this can be accounted for by the reduced output of neostigmine. By contrast, the excretion of the metabolite is not significantly affected by Cyanine 863 within 2 hr.

TABLE 1

EFFECT OF CYANINE 863 ON THE EXCRETION IN THE URINE OF THE RAT OF TOTAL RADIOACTIVITY, NEOSTIGMINE AND METABOLITE AFTER INTRAMUSCULAR INJECTION OF 25 μG OF [^{14}C]-NEOSTIGMINE

Treated animals were injected intramuscularly with Cyanine 863 1 hr before [^{14}C]-neostigmine. Excretion in the urine is expressed as a percentage of the dose of neostigmine. Figures are the means and standard deviations of four experiments with Cyanine 863 and five to seven control experiments. Values for P are for each time interval

Time after injection (min)	Excretion in urine (% of dose)					
	Total radioactivity ($P<0.05$)		Neostigmine ($P<0.01$)		Metabolite ($P>0.3$)	
	Treated	Control	Treated	Control	Treated	Control
15	8·4±2·6	14·7±1·8	7·2±2·8	13·5±2·1	0·8±0·3	1·2±0·8
30	16·3±0·9	24·1±2·0	13·3±0·9	20·4±1·8	3·1±1·3	3·7±1·7
45	22·4±3·6	31·5±2·2	16·3±2·0	24·5±1·8	6·6±2·5	6·9±2·2
60	27·1±5·0	37·2±2·3	17·7±2·7	26·3±1·8	9·7±3·8	10·9±2·0
120	36·4±7·0	48·4±4·3	19·1±3·3	28·5±2·2	20·2±6·8	20·0±5·0

Liver. The concentration of neostigmine and the metabolite in the liver was determined in three rats at various time intervals after intramuscular injection of 25 μg of [^{14}C]-neostigmine. The results in Table 2 show that within 10 min the concentration of radioactivity in

the liver accounted for 18% of the dose and that it reached its maximum within 30 min, after which it declined. These results agree closely with those already published (Roberts *et al.*, 1965). Almost all of the radioactivity was due to the metabolite and even 10 min after injection no more than a trace of neostigmine was detected. This evidence strongly suggests that the liver is intimately concerned in the metabolism of neostigmine and that the metabolite excreted in the urine probably arises from this source.

TABLE 2

TOTAL RADIOACTIVITY, NEOSTIGMINE AND METABOLITE IN RAT LIVER AFTER INTRAMUSCULAR INJECTION OF [^{14}C]-NEOSTIGMINE (25 μG)

Figures are individual values at each time interval. Total radioactivity is expressed as a percentage of the dose and neostigmine and metabolite as percentages of the total radioactivity in the liver

Time after injection (min)	Total radioactivity (%)	Metabolite (%)	Neostigmine (%)
10	17·8	98·4	1·6
30	25·2	98·6	1·4
60	18·3	99·4	0·6

These tentative conclusions led us to consider the effect of prior treatment of rats with SKF 525-A, a compound which inhibits drug metabolism in liver microsomes (Cooper, Axelrod & Brodie, 1954).

Previous treatment with SKF 525-A

The excretion of the metabolite and of neostigmine in the urine and their concentration in the liver were investigated in rats which were injected intraperitoneally with SKF 525-A (3.75 mg) 30 min before intramuscular injection of [^{14}C]-neostigmine.

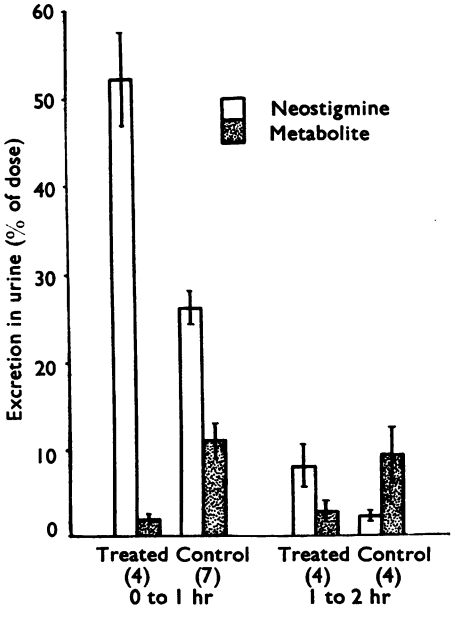

Fig. 3. Effect of SKF 525-A on the excretion of neostigmine and metabolite in rat urine after intramuscular injection of [^{14}C]-neostigmine (25 μg). Values are means and vertical lines are standard deviations. Number of animals in parentheses.

Urine. The mean output of neostigmine and the metabolite in urine was estimated 1 hr and 2 hr after injection of neostigmine. The results expressed as a percentage of the dose are shown in Fig. 3. Compared with the controls the output of the metabolite from the treated rats is significantly decreased while the output of neostigmine is increased. It is also noteworthy that in the treated rats neostigmine continues to be excreted at a greater rate than in the controls during the second hour. Estimation of the total radioactivity (Table 3) shows that the mean output of the treated rats was significantly greater than the control animals at both time intervals.

TABLE 3

EFFECT OF SKF 525-A ON THE EXCRETION OF TOTAL RADIOACTIVITY IN RAT URINE AFTER INTRAMUSCULAR INJECTION OF 25 μG OF [^{14}C]-NEOSTIGMINE

Treated animals were injected intraperitoneally with SKF 525-A (3·75 mg) 30 min before neostigmine. The output of radioactivity is expressed as a percentage of the dose. Values are means and standard deviations. Numbers of rats are in parentheses. $P<0·01$ for both time intervals

Time after [^{14}C]-neostigmine (hr)	Treated	Control
1	54·2±5·1 (4)	37·2±2·3 (7)
2	64·9±5·5 (4)	48·4±4·3 (4)

Liver. Three rats treated with SKF 525-A as described above were killed 30 min after intramuscular injection of [^{14}C]-neostigmine (25 μg). Total radioactivity, neostigmine and the metabolite were estimated in each liver. The results in Table 4 show that the proportion of the metabolite is substantially reduced when compared with results for untreated rats (Table 2). Furthermore, whereas only a trace of neostigmine could be detected in the control rats, about 80% of the radioactivity in the treated rats was due to unchanged neostig-

TABLE 4

RELATIVE PROFORTIONS OF METABOLITE AND NEOSTIGMINE IN LIVER FROM RATS PREVIOUSLY TREATED WITH SKF 525-A AND KILLED 30 MIN AFTER INTRAMUSCULAR INJECTION OF [^{14}C]-NEOSTIGMINE (25 μG)

Rats were injected intraperitoneally with SKF 525-A (3·75 mg) 30 min before neostigmine. Figures are percentages of total radioactivity in the liver

Rat no.	Percentage of total radioactivity in liver due to	
	Metabolite	Neostigmine
1	13·2	86·6
2	23·9	76·1
3	21·7	78·3

mine. There was no significant difference between the treated and untreated rats in respect to concentration of total radioactivity. The pharmacological actions seen after the standard dose of neostigmine were slightly exaggerated and more prolonged in the SKF 525-A-treated rats. Whereas in the control rats generalized muscle twitching occurred within 5 min of the injection and lasted 30 min, in the treated rats this usually lasted for about 1 hr; gross salivation was not observed at any time, though there was some increase in peristalsis which resulted in occasional defaecation of a soft stool.

DISCUSSION

These results show that the rapid excretion of radioactivity in urine after intramuscular injection of [^{14}C]-neostigmine which was reported by Roberts *et al.* (1965) is due almost entirely to neostigmine. The results also show that neostigmine is rapidly metabolized, and that a metabolite, which we have tentatively assumed to be *m*-hydroxyphenyltrimethylammonium, can be detected in the urine within 15 min. Thereafter the proportion of the metabolite increases and during the second hour the excretion of radioactivity is almost entirely due to the metabolite. The occurrence of the metabolite in the liver within 10 min of injection and the failure to detect more than 2% neostigmine leads us to conclude that the liver plays an important part in the metabolism of neostigmine and is probably the main source of the metabolite found in the urine. The experiments with SKF 525-A strongly support this conclusion in that the proportion of the metabolite detected in the liver was markedly reduced and that 80% of the total radioactivity was due to neostigmine. This is further emphasized by the low output of the metabolite in the urine and the increased output of neostigmine.

The fact that the output of neostigmine was also increased during the second hour emphasizes the relatively minor part played by plasma in metabolism of this drug. Indeed, repeated attempts to demonstrate metabolism by *in vitro* incubation of neostigmine with rat plasma for periods up to 1 hr were unsuccessful and underline the slow nature of such a process, as was previously noted by Nowell *et al.* (1962b) who used human plasma.

Since SKF 525-A is known to inhibit the metabolism of some drugs by liver microsomes, the work of Hodgson & Casida (1961) is particularly relevant. After incubating neostigmine *in vitro* with a rat liver microsome preparation they detected formaldehyde which they considered to be a product of the hydrolysis of one of the methyl groups on the carbamate side chain. They suggested that the metabolite formed during incubation was the *N*-methyl-*N*-hydroxymethyl carbamate ester of *m*-hydroxyphenyltrimethylammonium. Such a compound would inevitably undergo further hydrolysis to *m*-hydroxyphenyltrimethylammonium in the conditions which we used for separation of metabolite from neostigmine. It is clearly important to establish whether the hydrolytic product postulated by Hodgson & Casida (1961) is a precursor of the metabolite which we have detected in urine and liver.

The experiments with Cyanine 863 provide an interesting contrast to those with SKF 525-A. We have previously shown that prior or concurrent administration of Cyanine 863 inhibits the output of radioactivity in the hen and the rat after an intramuscular injection of [^{14}C]-neostigmine (Roberts *et al.*, 1965). The results now reported show that this cyanine dye inhibits the output of neostigmine but whether it also inhibits the tubular secretion of the metabolite is uncertain. Although the evidence shows that the output of the metabolite is not affected we do not consider the data sufficient to conclude that there is no inhibition of renal tubular secretion of the metabolite. The decreased output of neostigmine might be expected to result in an increased production of the metabolite but this would not be reflected in an increased output in urine if tubular secretion of the metabolite were inhibited. To prove this point it would be necessary to know the concentration of the metabolite in blood.

The results of this investigation are relevant to the clinical use of neostigmine. They agree with the evidence of Scott (1962) that in patients with myasthenia gravis about 50%

of a dose of neostigmine injected intramuscularly is excreted within 2 hr in the urine. Although Scott *et al.* (1962) detected the presence of a metabolite in the urine of myasthenic patients treated orally with neostigmine, they were unable to demonstrate it in the urine of patients who were given neostigmine by intramuscular injection. Since we have detected the metabolite in the urine of the rat after intramuscular injection of [^{14}C]-neostigmine it seems likely that this may also occur in man. The most likely explanation for this anomaly is the relative insensitivity of the method of Scott *et al.* (1962) for detecting the metabolite. From the early and prolonged occurrence of the metabolite we tentatively conclude that its action is an essential part of the therapeutic effect of neostigmine.

SUMMARY

1. The metabolism of neostigmine in the rat has been investigated using a standard intramuscular dose of 25 μg of [^{14}C]-neostigmine.

2. A method is described for the separation of neostigmine from a metabolite thought to be *m*-hydroxyphenyltrimethylammonium by paper electrophoresis.

3. Within 30 min of an injection of [^{14}C]-neostigmine about 20% of the dose is excreted as neostigmine and about 4% as metabolite. Thereafter the proportion of the metabolite increases and during the second hour the excretion of radioactivity is almost entirely due to metabolite.

4. Within 10 min of the injection approximately 98% of the radioactivity concentrated in the liver is due to the metabolite. Even when the peak concentration of radioactivity was reached at 30 min only 1.5% of neostigmine was detected.

5. Previous treatment with an inhibitor of renal tubular secretion, Cyanine 863, reduced the output of neostigmine but not of metabolite.

6. When rats had been previously treated with SKF 525-A the output of metabolite in the urine was significantly reduced and that of neostigmine was increased.

7. The concentration of total radioactivity in the liver of SKF 525-A-treated rats 30 min after neostigmine injection was not different from control animals but there was a substantial difference between the two groups in the proportion of metabolite and neostigmine. Whereas in the control group approximately 98% of radioactivity was due to the metabolite and only about 2% to neostigmine, in the treated group 20% was present as the metabolite and 80% as neostigmine.

8. We conclude that after intramuscular injection neostigmine is rapidly excreted and metabolized and that the liver plays an important part in its metabolism.

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