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METABOLISM OF NEOSTIGMINE *IN VITRO*

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Abstract—The metabolism of neostigmine by fractions of rat liver has been investigated using ^{14}C neostigmine. It has been shown that the drug is metabolized to 3-hydroxyphenyltrimethylammonium by the microsome fraction of rat liver and that the metabolism is completely inhibited by SKF 525-A. These results support the evidence from *in vivo* studies that the metabolism of neostigmine is inhibited by SKF 525-A. It is suggested that the clinical effects of neostigmine may be influenced by concurrent treatment with other drugs which are metabolized by the liver.

A METABOLITE of neostigmine was first detected in the urine of patients with myasthenia gravis after oral administration of the drug.[1] Subsequent studies with ^{14}C-neostigmine confirmed that a similar metabolite is excreted in the urine of rats after intramuscular and oral administration.[2, 3] The collective evidence indicated that the metabolite is probably 3-hydroxyphenyltrimethylammonium, and further recent work by Roberts *et al.*[4] has shown that in the rat, some of the metabolite is conjugated with glucuronic acid and excreted in the urine. Since high concentrations of metabolite were found in the liver[2] it was concluded that the liver plays an important part in the metabolism of neostigmine. This conclusion was strongly supported by the finding that prior administration of *N,N*-diethylaminoethanol ester of diphenylpropylacetate hydrochloride (SKF 525-A), a compound known to inhibit liver microsomal drug metabolism,[5, 6] considerably reduced the amount of neostigmine which was metabolized.[2]

The results described in this paper provide evidence that preparations of rat liver microsomes metabolize neostigmine *in vitro* to 3-hydroxyphenyltrimethylammonium.

MATERIALS AND METHODS

Chemicals

^{14}C-neostigmine iodide, obtained from the Radiochemical Centre, Amersham, had a specific activity of 15·2 μc/mg. Nicotinamide-adenine dinucleotide phosphate (NADP), reduced NADP ($NADPH_2$) and glucose-6-phosphate (G-6-P) were supplied by Sigma London Chemical Co. Ltd. and SKF 525-A was provided by Smith Kline & French Research Institute (Welwyn Garden City).

Preparation of liver fractions

Male rats of 150–200 g body wt. were killed by decapitation. The liver was immediately dissected out and immersed in an ice-cold solution of either 1·15% (w/v) KCl or 0·25M sucrose. The liver was then homogenized in 2 vol. 1·15% KCl or 9 vol. 0·25M sucrose using a Potter–Elvehjem homogenizer.

The homogenate was centrifuged for 15 min at 15,000 *g* in a Spinco 50 head at 4°; the supernatant containing microsomes and soluble enzymes was collected and the residue discarded.

In some experiments the supernatant was prepared with 0·25 M sucrose containing 0·2% nicotinamide and further centrifuged at 105,000 *g* for 1 hr. The residue thus obtained was resuspended in fresh sucrose-nicotinamide solution and again centrifuged at 105,000 *g* for 1 hr. The clear red microsomal pellet was resuspended in 0·1 M–Na_2HPO_4 adjusted to pH 7·4 with O·1M-KH_2PO_4 in the proportions of 1 ml buffer to 1 g equivalent of liver.

Incubation conditions and estimation of metabolism

Two main groups of observations were made using (a) the 15,000 *g* supernatant and (b) the 105,000 *g* suspended residue.

(a) *Experiments with the* 15,000 g *supernatant.* The incubation medium used was 0·65 μmole NADP, 1·0 μmole G-6-P, 50 μmole nicotinamide, 25 μmole magnesium chloride, 3 ml 0·1 M pH 7·4 phosphate buffer, 2 ml of the 15,000 *g* supernatant and 0·357 μmole (125 μg) ^{14}C-neostigmine iodide. The final volume was adjusted to 6 ml with water. Experiments without NADP were also carried out.

(b) *Experiments with the* 105,000 g *suspended residue.* The incubation medium used was 2μmole $NADPH_2$ or NADP, 0·4 ml 105,000 *g* residue in 0·1 M pH 7·4 phosphate buffer and 0.143 μmole (50 μg) ^{14}C-neostigmine iodide. The final volume was adjusted to 2 ml with water.

In all experiments the media were incubated at 37° for 30 min with continuous agitation in air. The reaction was stopped by the addition of 3 vol. ethanol. After settling overnight at room temperature the ethanol solution was centrifuged for 10 min at about 5000 rev/min to sediment the protein.

In each group of experiments an aliquot of each liver preparation was heated on a boiling water bath for 5 min and used as a control for non-enzymic hydrolysis.

The proportion of neostigmine metabolized was estimated by paper electrophoresis as described by Roberts *et al.*[2] It is noteworthy that in the present experiments only one metabolite peak was detected and it behaved similarly to concurrently run authentic samples of 3-hydroxyphenyltrimethylammonium.

The results were expressed as mμ mole of neostigmine metabolised in 1 hr by the equivalent of 1 g wet wt. of liver.

RESULTS

Table 1 shows the results obtained with the 15,000 g supernatant liver fraction with and without the addition of NADP. It can be seen that there is good agreement between the results obtained with homogenates prepared with KCl and with sucrose. It is also evident that the metabolism of neostigmine is considerably increased by the addition of NADP to the homogenates.

The fact that some metabolism occurred in the absence of added NADP can be explained by the probable presence of endogenous NADP in the liver fractions.

In one experiment SKF 525-A was added at a concentration of $4·8 \times 10^{-5}$ M. This resulted in complete inhibition of metabolism.

Experiments with the 105,000 *g* residue were designed to confirm that the site of metabolism of neostigmine is in the microsomal fraction, and that $NADPH_2$ is the essential co-factor. Table 2 shows that the addition of $NADPH_2$ to the buffer in which the 105,000 *g* residue was suspended resulted in considerable metabolism of neostigmine. In contrast no metabolism was detected when $NADPH_2$ was replaced by NADP.

The proportion of neostigmine metabolized in these experiments is less than was observed in the experiments with the 15,000 *g* supernatant. This can probably be attributed to the lability of $NADPH_2$ and the altered enzyme concentration rather than to metabolic activity in the supernatant (column 3).

TABLE 1. METABOLISM OF ^{14}C-NEOSTIGMINE BY THE 15,000 *g* SUPERNATANT FRACTION OF RAT LIVER

	Metabolism in mμ mole/g/hr	
	(A) Homogenized in 1·15% KCl	(B) Homogenized in 0·25 M sucrose
NADP added	216 ± 72 (6)	239 ± 37 (3)
No NADP	88 (1)	68 (1)
	79 (1)	22 (1)

^{14}C-neostigmine iodide (357 mμ mole) was incubated with the equivalent of (A) 0·67 g or (B) 0·2 g liver wet wt., fortified with nicotinamide, magnesium chloride and G-6-P in pH 7·4 phosphate buffer as described in the text. Where applicable values are expressed as means and S.D. Number of experiments are in parentheses.

TABLE 2. METABOLISM OF ^{14}C-NEOSTIGMINE BY THE 105,000 *g* FRACTION OF RAT LIVER

Metabolism in mμ mole/g/hr		
Residue		Supernatant
$+NADPH_2$	+NADP	$+NADPH_2$
111	0	0
144	0	–
138	–	–

^{14}C-neostigmine iodide (143 mμ mole) was incubated with the equivalent of 0·4 g liver wet wt. in pH 7·4 phosphate buffer as described in the text.

DISCUSSION

The evidence presented in this paper shows that neostigmine is metabolized by an $NADPH_2$ dependent liver microsome system. This is indicated in the first group of experiments with the 15,000 *g* supernatant fraction of liver. It is presumed that the NADP used in these experiments is reduced by glucose-6-phosphate dehydrogenase present in the supernatant fraction of rat liver.[7, 8] The experiments with isolated washed microsomes confirmed the specific requirement for reduced NADP.

The product of neostigmine metabolism appears to be 3-hydroxyphenyltrimethylammonium which is the metabolite that has been identified in rat and human urine after administration of neostigmine.[1–3] The fact that the addition of SKF 525-A completely inhibited metabolism is in agreement with the inhibition observed with this compound *in vivo* in earlier work.[2] The close agreement between results obtained *in vivo* and *in vitro* is regarded as substantial evidence in support of the view that the liver microsomes are largely responsible for the metabolism of neostigmine. The slow

rate of metabolism observed by Nowell *et al.*[9] when neostigmine was incubated with plasma provides further support for the conclusion that the liver is the most important site of metabolism of this drug.

The metabolism of a highly polar drug such as neostigmine by the liver microsome system is contrary to the theory, proposed by Gaudette and Brodie,[10] that only lipid soluble drugs are metabolized by this system. We investigated the distribution of neostigmine between 0·1 M pH 7·4 phosphate buffer and chloroform and found that only 1·2 per cent of the drug partitioned into the chloroform layer which is clear evidence of the highly polar nature of neostigmine. Mazel and Henderson[11] have also described the microsomal metabolism *in vitro* of two polar compounds. It would therefore appear to be desirable to investigate a wider range of polar compounds to obtain a better understanding of the physical properties of drugs which are metabolized by liver microsomes.

Hodgson and Casida[12] have suggested a metabolic pathway for dialkyl carbamates in which the first step is the oxidation of one of the terminal methyl groups to a labile methylol. The high pH of the electrophoresis buffer used in our experiments would probably cause a rapid breakdown of the methylol derivative to the phenolic compound, which was detected in our investigation.

The evidence of microsomal metabolism of neostigmine provided by these experiments may be of some relevance to the clinical use of the drug. As Cucinell *et al.*[13] have pointed out, the metabolism in experimental animals of a variety of drugs such as dicoumarol, phenytoin and antipyrine is accelerated as a result of chronic administration of phenobarbitone. They have also shown that phenobarbitone treatment in man decreases the plasma level of phenytoin and of dicoumarol. Thus it is possible that concurrent treatment with drugs such as barbiturates, or anticholinesterase compounds may substantially influence the response of patients with myasthenia gravis to neostigmine and of patients in whom the effects of tubocurarine require to be abruptly terminated.

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REFERENCES

1. C. A. SCOTT, P. T. NOWELL and A. WILSON, *J. Pharm. Pharmac.* **14**, 31T (1962).
2. J. B. ROBERTS, B. H. THOMAS and A. WILSON, *Br. J. Pharmac. Chemother.* **25**, 763 (1965).
3. J. B. ROBERTS, B. H. THOMAS and A. WILSON, *Biochem. Pharmac.* **15**, 71 (1966).
4. J. B. ROBERTS, B. H. THOMAS, M. A. HOSSAIN and A. WILSON, *J. Pharm. Pharmac.* **19**, 133 (1967).
5. J. AXELROD, J. REICHENTHAL and B. B. BRODIE, *J. Pharmac. exp. Ther.* **112**, 49 (1954).
6. J. R. COOPER, J. AXELROD and B. B. BRODIE, *J. Pharmac. exp. Ther.* **112**, 55 (1954).
7. G. E. GLOCK and P. MCLEAN, *Biochem. J.* **55**, 400 (1953).
8. R. W. NEWBURGH and V. H. CHELDELIN, *J. biol. Chem.* **218**, 89 (1956).
9. P. T. NOWELL, C. A. SCOTT and A. WILSON, *Br. J. Pharmac.* **19**, 498 (1962).
10. L. E. GAUDETTE and B. B. BRODIE, *Biochem. Pharmac.* **2**, 89 (1959).
11. P. MAZEL and J. F. HENDERSON, *Biochem. Pharmac.* **14**, 92 (1965).
12. E. HODGSON and J. E. CASIDA, *Biochem. Pharmac.* **8**, 179 (1961).
13. S. A. CUCINELL, A. H. CONNEY, M. SANSUR and J. J. BURNS, *Clin. Pharmac. Ther.* **6**, 420 (1965).