In vitro Synthesis of Metabolites of ^{14}C-Pyridostigmine

The main metabolite of pyridostigmine, in both experimental animals and in man, has been identified as 3-hydroxy-N-methyl-pyridinium[1-3]. Hydrolysis of the quaternary amine predominantly occurs in the soluble fraction of the liver cell, and is independent of the co-factor NADPH[4]. Nevertheless, clinical evidence suggests that 3-hydroxy-N-methylpyridinium may not be the only breakdown product of pyridostigmine, since small amounts of other metabolites have been detected in the urine of myasthenic subjects[2,3]. In the present experiments, an attempt was made to isolate other metabolites by incubation of ^{14}C-pyridostigmine with rat liver homogenates in in vitro conditions.

Materials and methods. Male Wistar rats (150-300 g body wt.) were housed at a temperature of 24 °C. Experimental animals were killed by decapitation, and rat liver homogenates and subcellular fractions were prepared as described elsewhere[4]. Protein in liver fractions was determined by standard methods[5]. Homogenates and subcellular fractions were then incubated at 38 °C with ^{14}C-pyridostigmine iodide or 3-hydroxy-N-methyl-^{14}C-pyridinium iodide; in some experiments, specimens were heated (100 °C for 1 min) prior to incubation.

Samples of the incubate were analyzed for possible metabolites by electrophoresis[3] and by paper chromatography. The radiochromatograms were resolved by the descending technique in solvent system 1 (isopropyl alcohol saturated with 0.1 *M* borate buffer, pH 9.2) and solvent system 2 (ethyl acetate:pyridine:water, 5:3:2 by vol.). Labelled metabolites on electrophoretograms or chromatograms were identified and counted by a Tracerlab radiochromatogram scanner.

In other studies, the labelled metabolites were eluted from paper chromatograms with warm water. Eluates (0.25 ml) were incubated (24 h at 38 °C) with β-glucuronidase (1,250 Fishman units/0.25 ml acetate buffer), sulphatase (10 units/0.25 ml acetate buffer), or acetate buffer alone (0.25 ml; 0.2 *M*, pH 5.0). The reaction was terminated by the addition of ethanol, any precipitate was removed by centrifugation, and the supernatant was resolved by chromatography in solvent systems 1 and 2.

Results. Approximately 25% of enzyme preparations catalyzed the formation of a radioactive metabolite that could be distinguished from 3-hydroxy-N-^{14}C-methylpyridinium. Between 1% and 10% of the total radioactivity in incubates was present as this additional metabolite of pyridostigmine. Although its electrophoretic mobility was similar to 3-hydroxy-N-methylpyridinium, eluates containing the additional metabolite could be distinguished by their characteristic Rf values in the two solvent systems used. The metabolite was not produced by heated homogenates and its formation was linear with time for a limited period (approximately 4 h); fractionation experiments suggested that catalytic activity was predominantly present in the soluble fraction of the liver cell (Table).

The additional metabolite probably contains a carbamate group, since it was not synthesized when 3-hydroxy-N-^{14}C-methylpyridinium was used as a substrate. Its electrophoretic mobility and Rf values in solvents 1 and 2 were unaffected by prior incubation with β-glucuronidase or sulphatase.

Discussion. The present results confirmed that 3-hydroxy-N-methylpyridinium was not the only metabolite of pyridostigmine. An additional metabolite, which could be distinguished from 3-hydroxy-N-^{14}C-methylpyridinium by its chromatographic behaviour, was identified by incubation of ^{14}C-pyridostigmine with rat liver fractions. Only 25% of enzyme preparations catalyzed the formation of the new metabolite; in the remaining 75% of cases, only 3-hydroxy-N-^{14}C-methylpyridinium was formed. It is possible that these differences are related to heterogenicity between experimental animals, although other explanations cannot be entirely excluded. The synthesis of the new metabolite was clearly dependent on an enzymic reaction, since it was not produced by heated homogenates, and its formation was linear with time for only a limited period. The additional metabolite of pyridostigmine may be related to one of the breakdown products of the quaternary amine previously identified in myasthenic urine[2,3].

A number of unsuccessful attempts have been made to determine the structure of the new metabolite. Although its UV- and IR-spectra differ from pyridostigmine and 3-hydroxy-N-methylpyridinium, insufficient was synthesized in vitro for NMR- and mass spectrum determinations. In future experiments, it is hoped to confirm the identity of the metabolite and to elucidate its possible clinical significance.

Zusammenfassung. Der Metabolismus von ^{14}C-Pyridostigmin wurde in Leberhomogenaten der Ratte untersucht. In vitro konnte die Synthese von zwei Pyridostigminmetaboliten festgestellt werden.

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Synthesis of the additional metabolite of pyridostigmine by different liver fractions

Liver fraction	Enzyme activity (nmol metabolite/mg protein/120 min)
Homogenate	6.8 ± 1.2
Supernatant (105,000 *g*)	20.0 ± 4.1
Microsomes	0

Values represent the mean ± S.E. of 5 experiments.

[1] R. D. N. Birtley, J. B. Roberts, B. H. Thomas and A. Wilson, Br. J. Pharmac. *26*, 393 (1966).
[2] P. Kornfeld, A. J. Samuels, R. L. Wolf and K. E. Osserman, Neurology *20*, 634 (1970).
[3] S. M. Somani, J. B. Roberts and A. Wilson, Clin. Pharmac. Ther. *13*, 393 (1972).
[4] P. A. Burdfield, T. N. Calvey and J. B. Roberts, J. Pharm. Pharmac. *25*, 428 (1973).
[5] O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, J. biol. Chem. *193*, 265 (1951).
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