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EXCRETION AND METABOLISM OF [^{14}C]-PYRIDOSTIGMINE IN THE RAT

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Although pyridostigmine is extensively used in the treatment of myasthenia gravis, there is little information available about its absorption, excretion and distribution. Nowell, Scott & Wilson (1962) showed that after oral administration to patients with myasthenia gravis a greater proportion of the daily dose of pyridostigmine was excreted in the urine than of neostigmine. They also reported that after withdrawing treatment the excretion of pyridostigmine in the urine continued for at least 2 days, whereas when treatment with neostigmine was stopped this drug was not detected in the urine after 24 hr. They suggested that pyridostigmine and neostigmine might differ also in their rates of metabolism but they were unable to provide evidence in support of this because the metabolites of these drugs could not be estimated by their assay procedure.

Recent work in these laboratories has shown that, after intramuscular injection of [^{14}C]-neostigmine in the rat and hen, it is rapidly eliminated by renal tubular secretion and rapidly metabolized in the liver (Roberts, Thomas & Wilson, 1963, 1965a,b). We have extended this work to a similar study of [^{14}C]-pyridostigmine.

METHODS

Pyridostigmine iodide labelled with ^{14}C in the methyl group of the quaternary nitrogen atom had a specific activity of 15 μc/mg and was supplied by the Radiochemical Centre, Amersham. It was used to examine the excretion and metabolism of pyridostigmine in rats injected intramuscularly with a standard dose of 100 μg in 0.1 ml. of water.

Rat. Male rats weighing 150 to 200 g, deprived of solid food overnight, were placed in metabolism cages (Bollman, 1948). They were hydrated by the administration of 5 ml./100 g of warm tap water by stomach tube. This was repeated 1 hr later. Pyridostigmine was injected intramuscularly into a hindlimb, and urine free from faeces was collected at 20-min intervals for 1 hr and then at 2, 3, 6 and 24 hr.

In the distribution studies rats were decapitated; blood was collected and the tissues were immediately dissected and weighed. They were estimated for radioactivity after extraction with trichloracetic acid by the method previously described (Roberts *et al.*, 1965a).

In experiments concerned with excretion of radioactivity as respiratory carbon dioxide, rats were housed in glass metabolism cages with water but no food (Gage, 1959, 1963). Respiratory carbon dioxide was collected in 10% aqueous sodium hydroxide solution and estimated for radioactivity by counting scintillations, using the scintillation fluid described by Kinard (1957).

Estimation of pyridostigmine and its metabolites

Pyridostigmine was separated from its metabolites in urine and liver extracts by paper electrophoresis and in some cases also by paper chromatography.

In these experiments the liver extracts were further evaporated to 1 ml., and 10 ml. of a 3% aqueous solution of sodium tetraphenylboron was added. The liquid was then centrifuged at 2,300 *g* for 5 min, the supernatant fluid was discarded and the solid residue was washed twice each with 10 ml. of water. The residue was dissolved in the minimum volume of acetone (1 to 2 ml.). The solution was then centrifuged to separate any insoluble residue; the supernatant fluid was pipetted into a 100 ml. centrifuge tube and to it was rapidly added 10 ml. of a 0.2% aqueous solution of silver nitrate. After centrifugation at 2,300 *g* for 5 min the supernatant fluid was collected and set aside. The solid residue was extracted again with acetone (1 to 2 ml.) and centrifuged. To the supernatant fluid 10 ml. of a 0.1% aqueous solution of silver nitrate was rapidly added; after centrifugation the supernatant fluid was collected and combined with the supernatant fluid previously set aside and evaporated to 0.5 ml. This concentrated solution was then subjected to paper electrophoresis and paper chromatography.

Paper electrophoresis

The method was similar to that used by Roberts *et al.* (1965b), except that the borate buffer was 0.05 M and the duration of electrophoresis was 50 min at 550 V and 0.4 mA/cm strip width.

Paper chromatography

Specimens of urine and aqueous extracts of liver were applied as a spot to Whatman No. 1 paper (45 × 3.5 cm) at a point 8 cm from one end, and dried by a current of hot air (Nowell *et al.*, 1962). The papers were arranged for descending development in a Shandon chromatography tank which contained an atmosphere saturated with the eluting solvent vapour at room temperature. After the papers had been equilibrated for 20 to 30 min in this atmosphere the eluting solvent system (methyl ethyl ketone:acetone:water:0.880 ammonia; 5:4:1:0.01) was introduced into the trough at the top of the tank by a tap funnel fixed into the lid of the tank. Development proceeded for 3 to 3.5 hr, and when the solvent front had travelled 30 cm beyond the origin the papers were removed from the tank and dried in a current of warm air after marking the position of the solvent front. Each paper was then cut transversely into serially numbered strips 1 cm wide and each strip was counted for radioactivity, using the method of Roberts *et al.*, 1965b).

Hen

Hens weighing 2.2 to 2.6 kg were anaesthetized and prepared for experiment by the method previously described (Roberts *et al.*, 1965a).

In three experiments 100 μg of [^{14}C]-pyridostigmine were injected intramuscularly in 0.1 ml. of distilled water into either the right or left leg. In another experiment it was administered as a continuous infusion of a solution containing 50 μg/ml. of [^{14}C]-pyridostigmine at a rate of 2.5 μg/min into the right saphenous vein into which a polyethylene cannula had been previously passed. Later in this experiment 1′-ethyl 2′-(3,6-dimethyl-2-phenyl-4-pyrimido) cyanine chloride (cyanine 863), which inhibits the renal transport of many quaternary ammonium compounds (Rennick, Kandel & Peters, 1956), was added to the pyridostigmine solution in a concentration of 1.27 mg/ml.

RESULTS

Excretion in urine

Rat. The radioactivity excreted in the urine of six rats, expressed as a percentage of the dose, is shown in Fig. 1. About 45% of the dose was excreted in the first hour and a further 17% during the next 2 hours. This initial rapid excretion of radioactivity strongly suggests that the drug is secreted by the renal tubules. Further evidence of this was obtained from experiments in the hen, which will be described below.

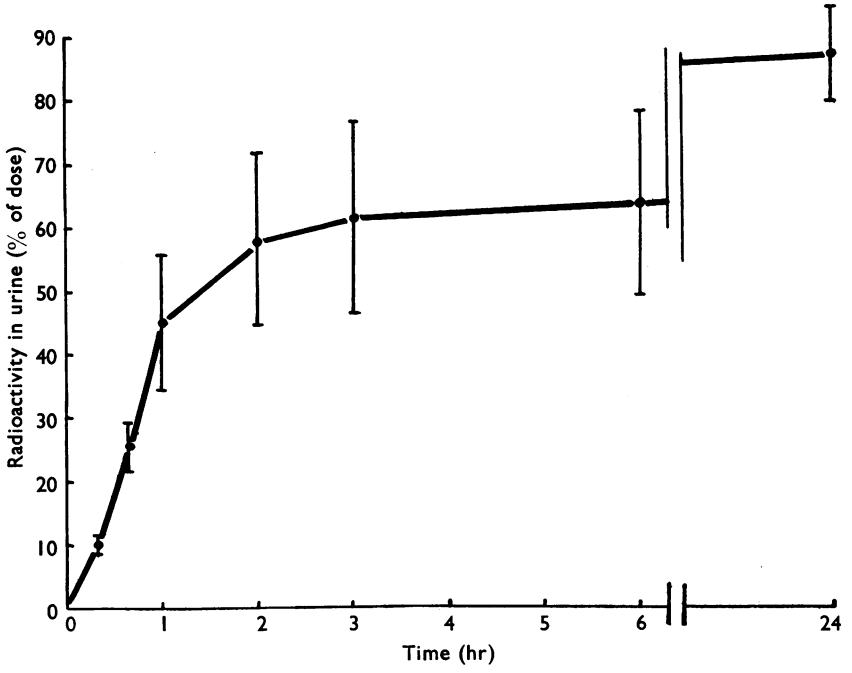

Fig. 1. Radioactivity excreted in the urine of the rat after intramuscular injection of [^{14}C]-pyridostigmine (100 μg). Each point is the mean of six experiments. The standard deviations are represented by the vertical lines.

Specimens of urine examined by paper electrophoresis and paper chromatography showed that the radioactivity separated into two distinct peaks, the positions of which coincided with concurrently run authentic specimens of pyridostigmine and 3-hydroxy-*N*-methylpyridinium. Fig. 2 is a typical example of the results with paper electrophoresis, and shows that the counts in strips numbered 12 to 16 are a measure of pyrido-

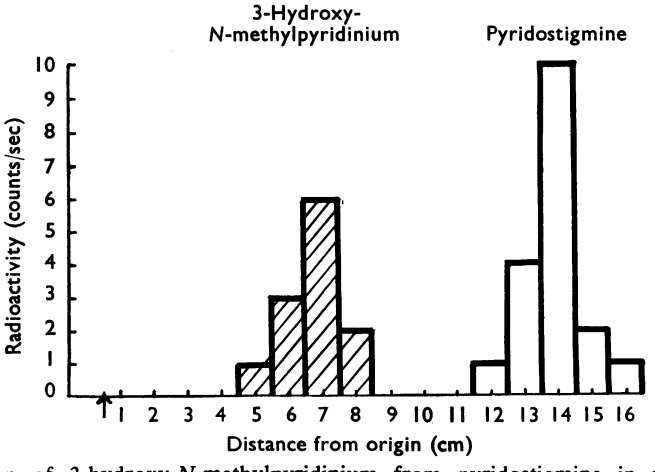

Fig. 2. Separation of 3-hydroxy-*N*-methylpyridinium from pyridostigmine in rat urine by paper electrophoresis. The shaded area (5 to 8 cm) represents radioactivity due to 3-hydroxy-*N*-methylpyridinium and the unshaded area (12 to 16 cm) that due to pyridostigmine.

stigmine, and those in strips 5 to 8 of 3-hydroxy-*N*-methylpyridinium. These peaks correspond to R_F values of 0.63 for pyridostigmine and 0.45 for 3-hydroxy-*N*-methylpyridinium obtained by paper chromatography.

The collective counts for each of the two zones were expressed as a percentage of the total count. From this it was possible to derive the proportions of metabolite and unchanged drug (pyridostigmine) which were excreted in the urine or contained in the liver.

The output of pyridostigmine and 3-hydroxy-*N*-methylpyridinium in the urine of six individual rats after intramuscular injection of [^{14}C]-pyridostigmine is shown in Fig. 3.

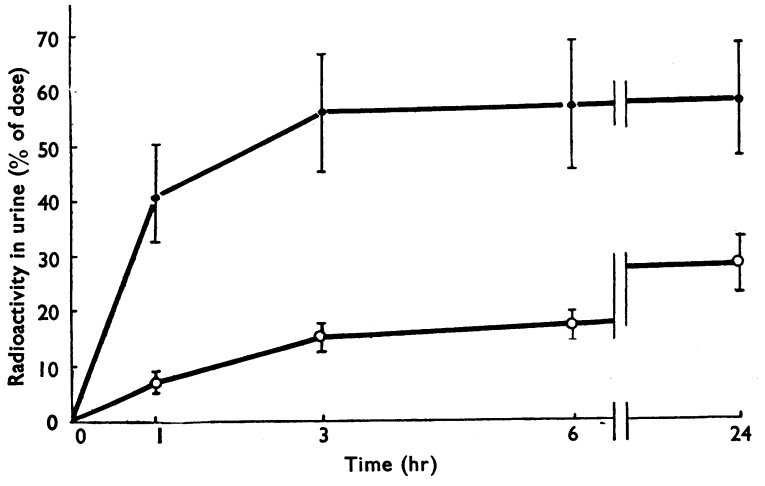

Fig. 3. Excretion of pyridostigmine (●) and metabolite (○) in the urine of the rat after intramuscular injection of [^{14}C]-pyridostigmine (100 μg). Each point is the mean of six experiments. The standard deviations are represented by the vertical lines.

Most of the radioactivity excreted during the first hour is pyridostigmine. Thereafter the output of pyridostigmine is slower and steadily decreases. By contrast, the excretion of the metabolite is initially slow and at 3 hr accounts for about 15% of the dose, after which its rate of excretion exceeds that of the unchanged drug.

Hen. The results of three experiments after intramuscular injection are summarized in Table 1, which shows that in each experiment the percentage of the dose excreted by

TABLE 1

EXCRETION OF RADIOACTIVITY BY IPSELATERAL AND CONTRALATERAL KIDNEYS OF THE HEN AFTER INTRAMUSCULAR INJECTION OF [^{14}C]-PYRIDOSTIGMINE

Hen no.	Dose of [^{14}C]-pyridostigmine (μg)	Duration of urine collection (min)	Radioactivity excreted by kidney (% of dose)		
			Ipselateral (*a*)	Contralateral (*b*)	Difference (*a*–*b*)
1	100	60	90·5	7·7	82·8
2	100	60	79·9	6·8	73·1
	100	60	57·4	14·9	42·5

the ipselateral kidney is greater than by the contralateral kidney. This strongly suggests that the drug was secreted by the renal tubules.

In another experiment pyridostigmine was infused at a constant rate of 2.5 μg/min into the saphenous vein. Urine was collected at intervals of 15 min for 1 hr. The syringe containing the pyridostigmine solution was changed to one containing the same concentration of pyridostigmine, together with cyanine 863 in a molar ratio of 1 : 20. Urine collection was continued for a further 1 hr. The differences between the mean values for the excretion of radioactivity by each kidney expressed as the percentage dose of pyridostigmine, before and during cyanine 863, were respectively 85.5 and 11.3. This indicates that pyridostigmine is excreted by the same renal tubular mechanism as neostigmine (Roberts *et al.*, 1965a).

Distribution in rat tissues

The radioactivity was estimated in a variety of tissues 30 min and 60 min after intramuscular injection of [^{14}C]-pyridostigmine. In each experiment two rats were used and the results are shown in Table 2. The most noticeable feature of these results is the high

TABLE 2

DISTRIBUTION OF RADIOACTIVITY IN RAT TISSUES 30 MIN AND 60 MIN AFTER INTRAMUSCULAR INJECTION OF 100 μG OF [^{14}C]-PYRIDOSTIGMINE

Rat weights: 1, 170 g; 2, 165 g; 3, 185 g; 4, 165 g

	Concentration of radioactivity (μg/g wet weight of tissue) at			
	30 min		60 min	
Tissue	Rat 1	Rat 2	Rat 3	Rat 4
Kidneys	8·11	10·13	0·37	0·36
Liver	1·16	1·15	0·43	0·47
Intestinal contents	0·57	0·73	0·42	0·40
Heart	0·42	0·55	0·80	0·91
Skeletal muscle	0·47	0·39	0·25	0·18
Spleen	0·25	0·21	0·22	0·25
Lungs	0·18	0·12	0·20	0·15
Blood	0·12	0·13	0·04	0·04
Skin	0·09	0·11	0·10	0·11
Thymus gland	nil	nil	nil	nil
Brain	nil	nil	nil	nil
Intestinal wall	nil	nil	nil	nil
Fat tissue	nil	nil	nil	nil

concentration of radioactivity in the kidney 30 min after injection ; this is probably related to the peak excretion of radioactivity in the urine (see Fig. 1). It is noteworthy that 60 min after the injection the concentration in the kidney had considerably decreased and was about the same as in the liver. Relatively high concentrations of radioactivity were detected in the intestinal contents at both time intervals. It is interesting to note that, whereas the concentrations in heart muscle and skeletal muscle were approximately similar 30 min after the injection, these values were quite different at 60 min. The concentration in skeletal muscle, as in most other tissues, decreased but in the heart it increased to about twice the previous level. No radioactivity was detected in the brain, intestinal wall, thymus gland and fat.

It is noteworthy that the levels of radioactivity in blood at each time interval are about one-tenth of those in the liver. This information is specially relevant to work on which

we had previously been engaged. The fact that the clinical effects of pyridostigmine after oral administration seem to be of longer duration than those of neostigmine raised the possibility of plasma protein binding of pyridostigmine. We investigated this problem by incubating rat plasma *in vitro* with [^{14}C]-pyridostigmine and by the use of plasma taken from rats 5 min after intramuscular injection of the drug. These specimens of plasma were examined by a variety of methods, including paper electrophoresis, gel filtration, dialysis, ion-exchange chromatography and ultraviolet spectroscopy, but in no case was there any evidence of protein binding of the drug. The results in Table 2 support this conclusion and were confirmed by estimating the radioactivity in blood specimens taken from six rats at different time intervals after intramuscular injection of [^{14}C]-pyridostigmine. Fig. 4 shows that the peak concentration of radioactivity occurred within 5 min of the injection and rapidly declined during 45 min ; only very low concentrations were detected after 1 hr.

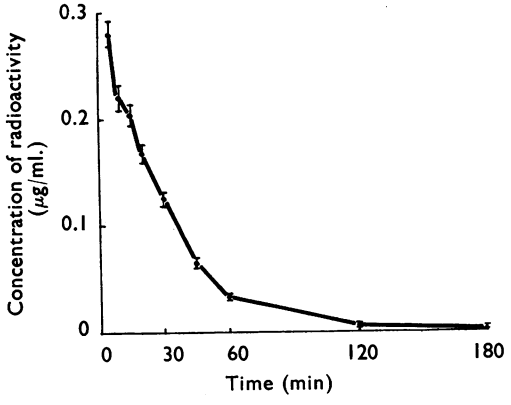

Fig. 4. Concentration of radioactivity in rat blood after intramuscular injection of [^{14}C]-pyridostigmine (100 μg). Each point is the mean of six experiments. The standard deviations are represented by the vertical lines.

Pyridostigmine and its metabolites in liver

The metabolism of pyridostigmine was further investigated in an experiment using twenty-one rats, each injected intramuscularly with [^{14}C]-pyridostigmine. At different time intervals after the injection three rats were killed, and extracts of each liver were estimated for total radioactivity, pyridostigmine and 3-hydroxy-*N*-methylpyridinium. The results in Fig. 5 show that the concentration of radioactivity in the liver reached its peak 20 min after the injection and rapidly decreased during the next 40 min. When the concentration of pyridostigmine reached its maximum in 20 min it accounted for about 70% of the radioactivity. The peak concentration of 3-hydroxy-*N*-methylpyridinium occurred a little later (30 min), then decreased more slowly, and from 45 min onwards exceeded that of pyridostigmine. These results suggest that the liver is probably the main site of metabolism of pyridostigmine and is the main source of the metabolite in the urine.

Paper chromatography of the extracts confirmed these results, and revealed also the presence of a third zone of radioactivity with an R_F of about 0.2. This was detected in

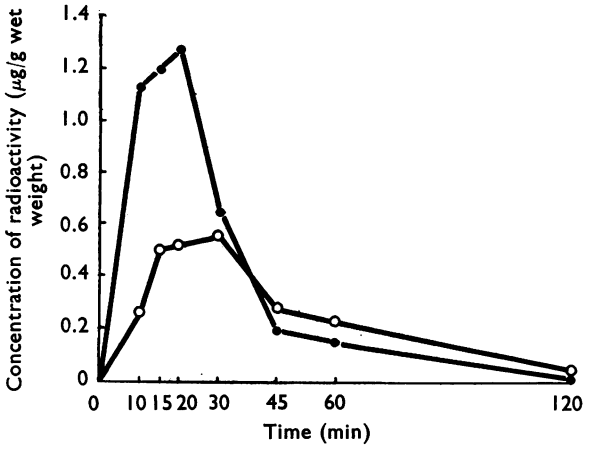

Fig. 5. Concentration of pyridostigmine (●) and of 3-hydroxy-*N*-methylpyridinium (○) in rat liver after intramuscular injection of [^{14}C]-pyridostigmine (100 μg). Each point is the mean of three experiments.

all extracts of liver obtained up to 30 min after injection, but the low concentrations of radioactivity in the later extracts did not permit its subsequent detection. We have tentatively concluded that this is a second metabolite of pyridostigmine, but have as yet been unable to establish its chemical nature.

Evidence of the metabolic release of the labelled methyl group

Five rats, which were individually housed in glass metabolism cages with water but no food, were injected intramuscularly with [^{14}C]-pyridostigmine. Urine, faeces, intestinal contents and respiratory carbon dioxide were collected for 24 hr and estimated for radioactivity. The results in Table 3 confirm that the main route for excretion of radioactivity was in the urine. The concentration in faeces and intestinal contents is substantially less than was detected 30 and 60 min after injection; this difference is probably due to

TABLE 3

EXCRETION OF RADIOACTIVITY IN RESPIRATORY CARBON DIOXIDE, FAECES, INTESTINAL CONTENTS AND URINE 24 HR AFTER AN INTRAMUSCULAR INJECTION OF 100 μG OF [^{14}C]-PYRIDOSTIGMINE

Radioactivities are expressed as percentages of the dose of pyridostigmine

Rat no.	Radioactivity (%) in: Respiratory carbon dioxide	Faeces and intestinal contents	Urine	Total
1	2·88	2·71	103·8	109·39
2	0·70	3·07	90·8	94·57
3	2·20	2·25	85·2	89·65
4	1·74	2·22	84·0	87·96
5	0·75	1·65	82·3	84·70
Mean and standard deviation	1·65±0·94	2·38±0·54	89·22±8·75	93·24±9·70

reabsorption from the alimentary tract. The radioactivity excreted as respiratory carbon dioxide accounted for up to about 3% of the dose and this suggests the possibility of either demethylation or more complete degradation of pyridostigmine.

DISCUSSION

The results of excretion studies in the rat have shown that after intramuscular injection pyridostigmine is rapidly absorbed and excreted in the urine. The rapid elimination of radioactivity in the urine, as shown in Fig. 1, is reflected by the initial high content of radioactivity in the kidneys. Experiments in the hen have shown that pyridostigmine is chiefly eliminated by renal tubular secretion, and this has been confirmed by the inhibitory effect of cyanine 863. These findings closely resemble those reported for neostigmine by Roberts *et al.* (1963, 1965a).

In the rat the radioactivity excreted in the urine has been shown to be pyridostigmine and 3-hydroxy-*N*-methylpyridinium. The proportion of the latter is lower than that of the corresponding metabolite in the urine of rats injected intramuscularly with neostigmine (Roberts *et al.*, 1965b). This suggests that pyridostigmine is more slowly metabolized than neostigmine. Both 3-hydroxy-*N*-methylpyridinium and pyridostigmine continue to be excreted up to 24 hr after the injection, at which time the metabolite represents about one-third of the total radioactiviy excreted in the urine.

About 10% of the dose is found in the alimentary tract but some of this appears to be reabsorbed, since only about 2 to 3% of the dose can be detected in the faeces and intestinal contents 24 hr after the injection. About 0.3% of the dose occurs in the bile during the 6 hr after injection ; we have as yet no evidence of the proportion of unchanged drug and of metabolite excreted in this way.

Distribution studies show that the radioactivity can be detected in most tissues except the brain, intestinal wall, fatty tissue and the thymus gland. The increase in concentration of radioactivity in heart muscle between 30 and 60 min after injection is surprising, and contrasts sharply with the reduction observed in other tissues, including skeletal muscle. The failure to detect plasma protein binding of pyridostigmine is supported by the rapid fall in the blood level of radioactivity after intramuscular injection of the drug.

The rapid rise in radioactive content of the liver is mainly due to the accumulation of pyridostigmine. There is also a consistent increase in the concentration of 3-hydroxy-*N*-methylpyridinium up to 30 min after the injection, by which time the pyridostigmine concentration has fallen considerably (Fig. 5). These results indicate that the liver is probably the main site for metabolism of the drug. In this respect it resembles neostigmine, but by contrast the rate of formation of the metabolite of neostigmine is very much faster (Roberts *et al.*, 1965b).

The detection of a third radioactive zone of low mobility in extracts of liver suggests the presence of a second metabolite. The fact that this was found in liver samples taken up to 30 min after the injection implies that it is rapidly formed. The failure to detect it after this time is probably due to the low content of radioactivity in the later specimens of liver. We have not yet found any evidence of the second metabolite in urine.

The occurrence of radioactivity in respiratory carbon dioxide suggests another metabolic pathway of pyridostigmine by release of the methyl group of *N*-methylpyridinium. This

has already been reported to occur with a number of other *N*-methylpyridinium compounds (McKennis, Bowman, Horvath & Bederka, 1964). These authors have pointed out that this kind of evidence does not necessarily exclude other interpretations, including more complete degradation of the molecule. Our results show that, whatever may be the explanation of this route of metabolism, it involves only a small proportion of the dose of pyridostigmine given by intramuscular injection.

SUMMARY

1. Pyridostigmine labelled with ^{14}C in the methyl group of the quaternary nitrogen atom has been used to study the mechanisms involved in the excretion, distribution and metabolism of pyridostigmine.

2. Methods are described for the separation of pyridostigmine from one of its metabolites, 3-hydroxy-*N*-methylpyridinium, by paper electrophoresis and paper chromatography.

3. In the rat, after an intramuscular dose, radioactivity is rapidly excreted in the urine, mostly as pyridostigmine; the excretion of 3-hydroxy-*N*-methylpyridinium steadily increases and after 3 hr is greater than that of pyridostigmine.

4. In the hen radioactivity is rapidly excreted by renal tubular secretion, which is inhibited by cyanine 863.

5. In the rat about 10% of the dose is present in the alimentary tract within 1 hr after injection, but some of this appears to be subsequently reabsorbed. About 0.3% of the dose is secreted in the bile.

6. A high concentration of radioactivity occurs in the kidney when excretion in the urine is at its maximum. Lower concentrations are present in the liver, intestinal contents, heart and skeletal muscle and blood. Radioactivity was also detected in the lungs, spleen and skin, but not in the brain, thymus gland, intestinal wall and body fat.

7. Radioactivity accumulates in the liver to a maximum concentration 20 min after injection, when it is mainly pyridostigmine. Thereafter the level of radioactivity steadily falls and proportionately greater amounts of 3-hydroxy-*N*-methylpyridinium are detected. A second metabolite was detected by paper chromatography, but its chemical nature has not yet been established. It is concluded that pyridostigmine is metabolized mainly in the liver.

8. The detection of radioactive respiratory carbon dioxide suggests that to a small extent pyridostigmine may be metabolized by another route.

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