Biochemical Pharmacology, 1962, Vol. 11, pp. 377–382. Pergamon Press Ltd., Printed in Great Britain.

METABOLIC STUDIES ON N-METHYLPYRIDINIUM-2-ALDOXIME—III. EXPERIMENTS WITH THE ^{14}C-LABELLED COMPOUND*

INGER ENANDER, ANDERS SUNDWALL and BO SÖRBO

Research Institute of National Defence, Dept. 1,
Sundbyberg, and Nobel Medical Institute, Biochemical Department, Stockholm, Sweden

(*Received* 15 *January* 1962; *accepted* 15 *January* 1962)

Abstract—N-methylpyridinium-2-aldoxime iodide (PAM), labelled with ^{14}C on the methyl group, was injected into rats and the excretion of the isotope in the urine was followed. Most of the radioactivity was excreted as unchanged PAM, but paper chromatography also demonstrated the formation of a number of metabolites, among which N-methylpyridinium-2-nitrile, N-methylpyridinium-2-carboxylic acid (homarine) and N-methyl-2-pyridone were identified.

IN PRECEEDING papers,[1, 2] studies on the metabolism of the nerve gas antidote N-methylpyridinium-2-aldoxime have been reported. This compound was found to be metabolized to N-methylpyridinium-2-nitrile, and to a small extent to thiocyanate. The investigations have now been extended to a study of the metabolism of the ^{14}C-labelled antidote, which has enabled us to identify two other metabolites.†

MATERIALS

Methyl-labelled N-*methylpyridinium-2-aldoxime iodide* (PAM) was prepared by quaternization[3] of pyridine-2-aldoxime with ^{14}C-labelled methyl iodide (4·9 mc/m mole). The reaction product was diluted with unlabelled PAM to give a specific activity of 1 mc/mmole. Paper chromatography of this product in different solvent systems demonstrated only one radioactive peak.

N-methyl-2-pyridone[4] and *N-methylpyridinium-2-carboxylic acid iodide* (homarine)[5] were synthesized by methods described in the literature. *N-methyl-α-picolinium amine diiodide* was synthesized from α-picoline amine by blocking the amino group with a phtaloyl group, quaternization with methyl iodide and liberation of the amino group by hydrazinolysis. M.p. = 200°C (d) ($C_7H_{12}I_2N_2$ requires C = 22·2, H = 3·19, I = 67·2, found C = 22·2, H = 3·70, I = 67·7). *N-methylpyridinium-2-carboxyamide iodide* was prepared from pyridine-2-carboxyamide[6] by quaternization with methyl iodide. M.p. = 174 °C ($C_7H_9IN_2O$, calc. C = 31·9, H = 3·44, I = 48·1, found C = 31·8, H = 3·49, I = 48·3).

METHODS

Rats (about 200 g) were kept in metabolism cages, and the 24-hr urine collected and diluted to 50 ml with water. In two experiments carbon dioxide in the expired air

* Communicated to the First International Pharmacological Meeting, in Stockholm, August, 22–25 1961.

† The formulae of the metabolites and of the presumed intermediates are shown in Fig. 2.

was also collected and assayed for ^{14}C by standard techniques.[7] Paper chromatography was run on Whatman no. 1 with descending development. The chromatograms were assayed for radioactivity in a Frieseke and Hoepfner paper chromatograph FH 52, with two G-M tubes FH 215a as detectors. Reversed isotope dilution of the pyridone was carried out in the following way. Carrier compound (about 200 mg) was added to 5 ml urine, and the mixture was extracted with chloroform. The chloroform phase was evaporated and pyridone picrate isolated from the residue. Constant specific activity was reached after four recrystallisations. A similar technique was used in case of PAM and N-methyl-α-picolinium amine diiodide, but the picrates were in these cases isolated directly from urine. Radioactivity determinations were made in a Frieseke and Hoepfner windowless flowcounter FH 407, with appropriate corrections for self-absorption, when necessary.

RESULTS

If the metabolic fate of a labelled compound has to be followed, for instance by paper chromatography, it is essential that the labelled group remains attached to the backbone of the compound during the metabolic transformations. In order to test for any demethylation *in vivo* of PAM, two rats were injected intramuscularly with 40 μC (40 mg/kg body weight) of the labelled compound and respiratory carbon dioxide collected for 6 hr. Only 0·02 and 0·06 per cent respectively of the injected ^{14}C was found in this fraction, and thus very little demethylation had taken place.

When the urinary excretion of ^{14}C was followed after injection of tagged PAM it was found that 80–90 per cent of the injected radioactivity after intramuscular administration was excreted within 24 hr (Table 1). Paper chromatography was then

TABLE 1. RADIOACTIVITY EXCRETED IN RAT URINE AFTER ADMINISTRATION OF ^{14}C-LABELLED PAM

Route of administration	Dose (mg/kg)	Days after administration				
		1	2	3	4	5
intramuscular	40	91	1	1	—	—
intramuscular	40	87	2	2	—	—
intramuscular	100	84	6	2	1	0·1
per os	100	52	6	4	2	0·2

Figures given represent per cent of the injected dose.

carried out on this urine, but only neutral and acid solvent systems were explored since PAM is unstable in alkaline solution. The best separation was obtained in *n*-butanol:acetic acid:water (4:1:1). When urine was chromatographed in this system, usually five peaks were observed with the radiochromatograph (Fig. 1). After one week exposure autoradiography gave the same result, but when the exposure time was extended to four weeks an additional number of spots appeared. As they evidently represented a very small fraction of the original dose, they were not explored

further. As shown in Fig. 1, the compound was more extensively metabolized when administered by the oral route. It was established that if ^{14}C-PAM was incubated *in vitro* with rat urine for 24 hr at room temperature, no significant transformation of the compound was detected The metabolites found in the experiments described are thus formed in the body of the animal. The major component shown in Fig. 1 (indi-

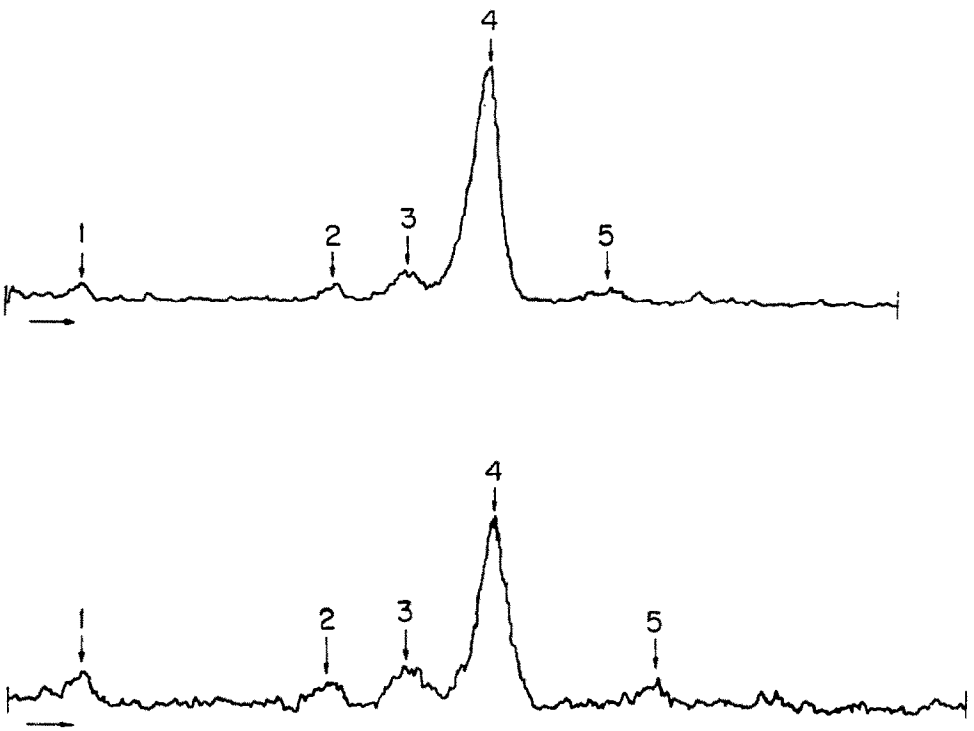

FIG. 1. Radiochromatography of urine from ^{14}C-PAM treated rats. Solvent system *n*-butanol : acetic acid : water. Direction of flow indicated by arrow. Dose of PAM: 40 mg/kg body weight. Upper curve: Intramuscular injection. Lower curve: Administration *per os*.

cated by "4") had an R_f-value of 0·53, identical with that of PAM. Reversed isotope dilution also confirmed that 80–90 per cent of the radioactivity in the urine excreted in the first 24-hr period after intramuscular injection was present as the parent compound. Another component in the chromatogram with an R_f-value of 0·44 (indicated by "3") was immediately identified from previously published results as N-methylpyridinium-2-nitrile.[2] A third component ("2") had an R_f-value of 0·35, identical with that of an authentic specimen of N-methylpyridinium-2-carboxylic acid (homarine).[8] The identity could in this case not be established by reversed isotope dilution, as no crystalline derivate of homarine, suitable for this purpose, could be found. Identification was instead accomplished by paper chromatography. The presumed homarine spot was eluted and rechromatographed in other solvent systems together with carrier homarine. The latter was localized by its strong ultra-violet absorption. In the two solvent systems 5 per cent KH_2PO_4 saturated with iso-amylalcohol[9] and 95 per cent ethanol:conc. ammonia (19:1)[10] the homarine spot and the radioactivity

coincided. The latter system resolved homarine from N-methylpyridinium-2-carboxyamide, in contrast to the *n*-butanol:acetic acid:water (and the KH_2PO_4) system. No formation of the amide could thus be demonstrated.

When urine was chromatographed in *n*-butanol:acetic acid:water, a spot with an R_f-value of around 0·10 appeared (indicated by "1" in Fig. 1). N-methyl-α-picolinium amine was found to have the same R_f-value in this solvent system, but reversed isotope dilution showed that less than 0·5 per cent of the injected radioactivity was excreted as the amine. Therefore, the identity of peak "1" could not be established. Another peak with an R_f-value of around 0·70 (indicated by "5" in Fig. 1) was found to have approximatively the same R_f-value as "metabolite X" reported in a preceeding paper.[1]*

As it is known that PAM spontaneously decomposes to N-methyl-2-pyridone *in vitro*,[11] this compound was also searched for in urine from PAM treated animals. The pyridone was found to have an R_f-value of around 0·80, in agreement with reports in the literature[12] where a solvent system similar to ours was used. No radioactive spot with this R_f-value could, however, be detected when urine from ^{14}C-PAM treated rats was chromatographed. But it was observed that N-methyl-2-pyridone is rather volatile, and that a large fraction of the compound, applied to a paper chromatogram, escaped from the latter. However, the presence of this compound in the urine could be demonstrated and determined by reversed isotope dilution. About 5 per cent (range 3·5–9·0 in 3 experiments) of the injected radioactivity was recovered as pyridone-picrate in the urine from rats which had received 40 mg ^{14}C-PAM/kg by intramuscular injection.

DISCUSSION

It is shown in this paper that 0·02–0·06 per cent of the radioactivity of ^{14}C-PAM was expired as $^{14}CO_2$ in a 6-hr period. Available data[13, 14] indicate that about 50 per cent of a metabolic labile methyl carbon appears as CO_2, and if a linear relationship between time and demethylation is assumed, our data suggest that less than 0·5 per cent of the injected PAM was demethylated during a 24-hr period.

From this and previous work the following three metabolites of PAM have been identified: N-methylpyridinium-2-nitrile, homarine, N-methyl-α-pyridone and thiocyanate. N-methylpyridinium-2-nitrile has also been reported as a metabolite from PAM by others[15] although no details were given. Possible metabolic pathways leading from PAM to the metabolites are shown in Fig. 2. The oxime is converted to the nitrile by a reaction which is formally a dehydration. As the nitrile spontaneously decomposes to pyridone and cyanide in alkaline solution[11] it is reasonable to assume that the nitrile is a precursor of the pyridone *in vivo* as well. Nitriles may be converted to the corresponding carboxylic acids by hydrolysis with amides as intermediates,[16] and this suggests that the nitrile may be a precursor of homarine as well. The fact that homarine could not be detected among the alkaline hydrolysis products of PAM[11] does not exclude the possibility that enzymes which can accomplish this conversion may exist.

Two enzymic pathways however, could lead to homarine starting from PAM. One consists of a transoximation[17] giving the corresponding aldehyde, which may then

* In this experiment, urine treated with silver nitrate[1] was used. The "metabolite X" was localized on the chromatogram by cutting the latter into strips, which were assayed by thiocyanate analysis according to method 1.[1]

be oxidized to homarine by any of the enzymes (aldehyde oxidase, aldehyde dehydrogenase and xanthine oxidase) known to oxidize aldehydes to carboxylic acids. During this work we prepared N-methylpyridinium-2-aldehyde iodide and found that it had R_f 0·60–0·65 in the butanol:acetic acid: water system. No radioactive spot with this R_f-value could, however, be detected when the urine was chromatographed. Another possible pathway would be a rearrangement of the oxime to the amide and hydrolysis of the latter by amidase to homarine. However, no evidence for enzymes catalysing the conversion of oximes to amides has been found in the literature and no amide was detected in this work in urine from PAM-treated animals. Experiments with tissue preparations *in vitro* are evidently necessary in order to elucidate the biochemical reactions by which homarine is formed by PAM. Such studies have been started in this laboratory.

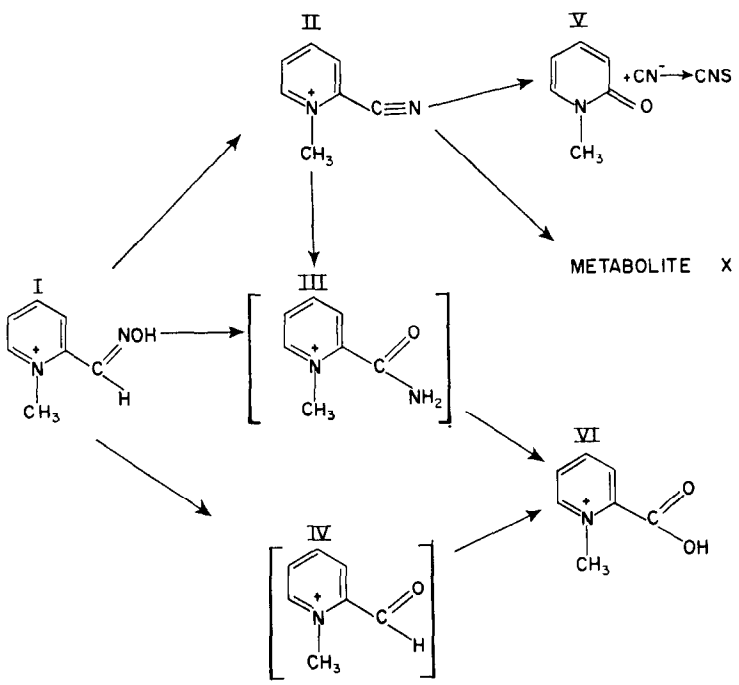

FIG. 2. Metabolic transformations of PAM.
I: N-methylpyridinium-2-aldoxime. II: N-methylpyridinium-2-nitrile. III: N-methylpyridinium-2-carboxyamide. IV: N-methylpyridinium-2-aldehyde. V: N-methyl-2-pyridone. VI: N-methylpyridinium-2-carboxylic acid.

Of interest in this connection is that Yamafugi *et al.*[19] have reported that certain oximes may be enzymically reduced to the corresponding amine. From the results presented in this paper it appears as if this reaction does not occur to any significant degree in case of PAM.

Finally, the question arises if any of the metabolites of PAM could contribute to the toxicity of the latter. The toxicity of the nitrile was previously reported and discussed.[2] We have also determined the LD_{50} of N-methyl-α-pyridone and homarine in

mice after intraperitoneal injection and obtained values about 400 and 900 mg/kg body weight respectively. As PAM is considerably more toxic (about 4 to 8-fold) the metabolites cannot be responsible for toxic side effects of the oxime.

REFERENCES

1. I. ENANDER, A. SUNDWALL and B. SÖRBO, *Biochem. Pharmacol.* **7**, 226 (1961).
2. I. ENANDER, A. SUNDWALL and B. SÖRBO, *Biochem. Pharmacol.* **7**, 232 (1961).
3. I. B. WILSON and S. GINSBURG, *Biochim. Biophys. Acta* **18**, 168 (1955).
4. E. A. PRILL and S. M. MCELVAIN, In A. H. BLATT, *Organic Synthesis Col. Vol. II*, John Wiley and Sons, Inc., N.Y., 1943, p. 419.
5. R. TURNAU, *Monath. Chem.* **26**, 530 (1905).
6. A. ENGLER, *Chem. Ber.* **27**, 1786 (1894).
7. R. W. COWGILL and A. B. PARDEE, *Experiments in Biochemical Research Techniques.* John Wiley and Sons, Inc., N.Y., 1957, p. 151.
8. D. ACKERMANN, *Hoppe-Seylers Z. physiol. Chem.* **295**, 1 (1953).
9. H. M. KALCKAR, J. L. STROMINGER and N. R. GEVIRTZ, *Biol. Bull.* **105**, 391 (1953).
10. H. M. BREGOFF, E. ROBERTS and C. C. DELWICKE, *J. Biol. Chem.*, **205**, 565 (1953).
11. R. I. ELLIN, *J. Amer. Chem. Soc.* **80**, 6588 (1958).
12. R. I. ELLIN and D. E. EASTERDAY. *J. Pharm. Pharmacol.* **13**, 370 (1961).
13. C. G. MACKENZIE, U. P. CHANDLER, E. B. KELLER, J. R. ROCHELE, N. CROSS and V. D. VIGNEAUD, *J. Biol. Chem.* **180**, 99 (1949).
14. N. PLOTNIKOFF, E. ELLIOTT and L. WAY, *J. Pharm. Exp. Ther.* **104**, 377 (1952).
15. J. L. WAY, H. TONG and R. RABIDEAU, *Fed. Proc.* **19**, 180 (1960).
16. R. B. WAGNER and H. D. ZOOK, *Synthetic Organic Chemistry*, John Wiley and Sons, Inc., N.Y., 1953, p. 412 and 570.
17. K. YAMAFUGI, S. HATANO, A. IDA, H. OMURA and S. MIZOGUCHI, *Enzymologia* **22**, 238 (1960).
18. L. FIELD, P. B. HUGHMARK, S. H. SHUMAKER and W. S. MARSHALL, *J. Amer. Chem. Soc.* **83**, 1983 (1961).
19. K. YAMAFUGI, *Enzymologia* **17**, 110 (1954).