Biochemical Pharmacology, 1966, Vol. 15, pp. 599–605. Pergamon Press Ltd., Printed in Great Britain.

METHYLATION OF DESMETHYLIMIPRAMINE BY RABBIT LUNG *IN VITRO**

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(*Received* 13 *August* 1965; *accepted* 1 *November* 1965)

Abstract—Studies of the methylation of analogs of imipramine by N-methyltransferase are described. Desmethylimipramine and nortriptyline, both secondary amines, are rapidly methylated by an enzyme in the soluble fraction of rabbit lung. Desdimethylimipramine, a primary amine, is slowly methylated by this enzyme. Imipramine, although not a substrate for the transferase, enhances the methylation of an endogenous substrate.

ADMINISTRATION of imipramine to rats counteracts a number of the effects, including sedation, evoked by reserpine and various benzoquinolizines.[1] The sedative effects of reserpine are prevented only when brain contains little or no imipramine and high levels of a metabolite, desmethylimipramine (DMI).[2, 3] This metabolite, which is formed from imipramine by the removal of one methyl group from the side chain nitrogen, is far more potent than imipramine in preventing the effects of reserpine.[4, 5] It thus appears that the antisedative action of imipramine is mediated through this metabolite. Imipramine and DMI, however, evoke little antisedative action in rabbits. This species difference in the pharmacologic action of imipramine and DMI appears to result from differences in the rate of metabolism in rats and rabbits.[6] For example, DMI is metabolized more rapidly than imipramine in rabbits but has a considerably longer half-life than the parent compound in rats.

Axelrod[7] has shown that the methylation of a number of amines is catalyzed by an enzyme present in rabbit lung. Since DMI rapidly accumulates in lung after its administration, these studies were undertaken to determine whether methylation of DMI in this organ could contribute to its rapid metabolism in the rabbit. Evidence presented in this paper shows that the methylation of DMI to imipramine is catalyzed by an enzyme present in the soluble fraction of rabbit lung.

METHODS

Enzyme preparation. The preparation of tissue samples was carried out at 0° to 4°. Adult male albino rabbits and male Sprague-Dawley rats were killed by a blow on the head. Lungs were removed and homogenized with 3 volumes of 1·15% (isotonic) KCl solution in a motor-driven mincer-grinder (Kontes Glass Co.). The homogenate was centrifuged at 50,000 *g* for 20 min, and the supernatant fraction containing the transferase enzyme was removed and stored at 4°.

* Supported by NIMH Grant 5 T1 MH-8107 from the United States Public Health Service.

Reaction mixtures were incubated in 15-ml glass-stoppered centrifuge tubes for 1 hr at 37° under air. A typical incubation mixture consisted of 100 μliters enzyme preparation, 100 μliters 0·5 M phosphate buffer (pH 7·9), 1 μmole substrate, and 7·5 mμmoles (3·7 $\times$ 10^4 dpm) S-adenosylmethionine-$^{14}CH_3$ in a final volume of 300 μliters.

Measurement of methylation. The N-methyl-^{14}C product was extracted from the incubation mixture into isoamyl alcohol at pH 10. The radioactivity extracted into the organic solvent was measured in a scintillation spectrometer after the addition of phosphor. Details of the procedure have been reported.[7] Although only a small amount of S-adenosylmethionine-$^{14}CH_3$ is extracted by this procedure, a sizable blank may result from the methylation of endogenous material. In several experiments this blank was eliminated by extraction of imipramine N-methyl-^{14}C from alkalinized incubation mixtures into heptane. The imipramine N-methyl-^{14}C was returned to an aqueous phase by extraction with 0·1 N HCl. The aqueous phase was made alkaline and the product extracted into isoamyl alcohol for counting. The procedure was as follows: 0·5 ml of 0·5 M borate buffer (pH 10) and 7 ml of heptane containing 1·5% isoamyl alcohol were pipetted into a glass-stoppered centrifuge tube containing the incubation mixture. The tube was shaken for 20 min, centrifuged, and 5 ml of the organic phase transferred to a centrifuge tube containing 1·5 ml of 0·1 N HCl. The tube was shaken for 20 min, centrifuged, and the aqueous phase transferred to a centrifuge tube and alkalinized to about pH 10 by the dropwise addition of 1·0 N NaOH. Isoamyl alcohol (6·0 ml) and 0·5 ml of pH 10 borate buffer were added and the tube shaken for 10 min. The tube was centrifuged and 4·0 ml of the organic phase transferred to a vial containing 3 ml of ethanol and 10 ml of phosphor (0·4% 2,5-diphenyloxazole and 0·01% 1,4-di[2,5-phenyloxazole] benzene in toluene). The radioactivity was measured in a scintillation spectrometer.

Paper chromatography. Samples dissolved in absolute ethanol were applied to Whatmann 3 MM paper, impregnated with peanut oil. The chromatograms were developed by ascending chromatography with concentrated ammonia: methanol (2:3) as the solvent system. After drying the chromatograms overnight in a fume hood, the spots were visualized by cooling the paper with liquid nitrogen and irradiating it with a short-wavelength u.v. lamp.[6] The chromatograms were then cut into 1-inch strips and these scanned for radioactivity in a paper scanner.

S-Adenosylmethionine-$^{14}CH_3$ was obtained from the New England Nuclear Corp. Samples of imipramine-^3H, imipramine, desmethylimipramine, desdimethylimipramine, nortriptyline, and S-adenosylmethionine were supplied through the courtesy of the Geigy Chemical Corp. and Dr. J. Axelrod.

Imipramine-^3H used in these studies was labeled on carbon atoms 10 and 11. The radio purity of this preparation was determined by paper chromatography in two solvent systems and found to be in excess of 99·5%.

RESULTS

Metabolism of DMI by rabbit lung. Desmethylimipramine and serotonin were incubated with S-adenosylmethionine-$^{14}CH_3$ and various cell fractions of lung. All the transferase activity for both substrates was found in the soluble supernatant fraction (Table 1). Moreover, the activity of the soluble fraction was destroyed by heating for 5 minutes at 90°. These results thus indicate that the methylation of DMI is catalyzed

by a transferase in lung with an intracellular localization similar to that described by Axelrod[7] for the nonspecific N-methyl transferase.

Isolation and identification of imipramine from incubation mixtures. DMI (20 μmoles) was incubated for 90 min with forty times the amount of soluble fraction of rabbit lung and other components described in Methods. The incubation mixture was

TABLE 1. INTRACELLULAR LOCALIZATION OF THE ENZYME IN RABBIT LUNG

Subcellular fraction	Radioactivity* Serotonin (dpm)	DMI (dpm)
600 *g* supernatant	6252	2100
9000 *g* supernatant	8263	3278
50,000 *g* supernatant	8829	4665
100,000 *g* supernatant	9007	4099
100,000 *g* pellet	588	23

* Radioactivity extracted directly into isoamyl alcohol; values are corrected for endogenous methylation. Various cell fractions equivalent to 25 mg rabbit lung were incubated with 100 μliters 0·5 M phosphate buffer (pH 7·9) 1 μmole substrate, and 7·5 mμmoles S-adensosylmethionine-$^{14}CH_3$; 5×10^3 dpm is equivalent to approximately 1 mμmole of product formed.

alkalinized by the addition of 2 ml of 1 N NaOH. Imipramine and unchanged DMI were extracted into 30 ml of heptane containing 1·5% isoamyl alcohol. The heptane extract was washed twice with 1/3 volume of 0·2 M phosphate buffer (pH 5·9) to remove most of the unchanged DMI. The heptane extract was evaporated to dryness under reduced pressure and the residue dissolved in 1·0 ml of absolute ethanol. The ethanol solution was evaporated to about 0·2 ml and applied to Whatmann 3 MM paper, impregnated with peanut oil, and the chromatogram subjected to ascending chromatography as described in Methods. Cooling the paper with liquid nitrogen and irradiating it with u.v. light made visible a fluorescent spot with an R_f value (0·12) corresponding to imipramine and a spot with an R_f value (0·29) corresponding to DMI. On scanning the chromatogram for radioactivity, the peak radioactivity was found to correspond to the imipramine spot. No radioactivity could be detected on chromatograms of incubation mixtures from which DMI had been omitted. The spots corresponding to imipramine and DMI were isolated and extracted with 5 ml of 0·01 N HCl. A 2-ml aliquot of the acid extract was made alkaline with 0·3 ml of 3 N NaOH and the fluorescence spectra of the solutions determined in a spectrophotofluorometer. The fluorescence characteristics of the material recovered from the spots resembled those of imipramine and DMI (activation maximum, 295 mμ; fluorescence maximum, 405 mμ, uncorrected). These solutions were extracted with 20 ml of heptane containing 1·5% isoamyl alcohol. A 5-ml aliquot of the organic phase was evaporated to dryness under reduced pressure and the residue dissolved in 1 ml of absolute ethanol. The radioactivity in the ethanol was determined in a scintillation spectrometer after the addition of phosphor. A large amount of radioactivity was found in the sample obtained from the imipramine spot; no radioactivity was present in the DMI spot.

Effect of substrate concentration. Various concentrations of DMI or serotonin were incubated for 1 hr with the soluble fraction of rabbit lung and other components described in Methods. With DMI as the substrate, the maximal rate of methylation was observed at a concentration of about $1{\cdot}7 \times 10^{-3}$ M (Table 2). The rate of methylation of serotonin, however, was enhanced by the addition of substrate in excess of $3{\cdot}3 \times 10^{-3}$ M. It is thus evident that a higher concentration of serotonin than of DMI is required to saturate the transferase enzyme.

TABLE 2. EFFECT OF SUBSTRATE CONCENTRATION

Concentration of substrate (M)	Radioactivity* Serotonin (dpm)	DMI (dpm)
$3{\cdot}3 \times 10^{-3}$	6982	4532
$1{\cdot}7 \times 10^{-3}$	5041	4471
$8{\cdot}3 \times 10^{-4}$	3152	3957
$4{\cdot}3 \times 10^{-4}$	2247	2322
$1{\cdot}7 \times 10^{-4}$	1151	1058
$8{\cdot}0 \times 10^{-5}$	662	544

* Radioactivity extracted directly into isoamyl alcohol. Values are corrected for endogenous methylation. Soluble fraction equivalent to 25 mg rabbit lung was incubated with substrate, 100 μliters 0·5 M phosphate buffer (pH 7·9), and 7·5 mμmoles S-adenosylmethionine-$^{14}CH_3$. The values are typical of 3 experiments.

Methylation of DMI in other tissues. Other rabbit tissues were examined for the ability to methylate DMI. Although the activity was highest in the lung, DMI was also methylated by the soluble fraction of heart and liver. The activity per gram in these tissues was about 40% of that in lung. No methylation of DMI could be measured in preparations of kidney or brain.

Methylation of related compounds. The enzyme in the soluble fraction of lung methylated nortriptyline, the secondary amine analog of amitriptyline, at about the same rate as DMI (Table 3). Desdimethylimipramine (DDMI), a primary amine, was slowly methylated in this system. Interestingly, when imipramine was incubated with the lung enzyme system, a radioactive product was formed which was extractable into isoamyl alcohol (Table 3). This metabolite, however, was not extractable into heptane (Table 4). In contrast, when DMI was incubated with the enzyme preparation, the amount of radioactivity extracted into heptane was nearly equal to that recovered in isoamyl alcohol. From these results it was obvious that the product formed in the presence of imipramine was considerably more polar than the drug itself. These findings suggested that imipramine had either undergone methylation to the quaternary amine or had in some manner stimulated the methylation of an endogenous amine. Of these possibilities the latter seemed more likely, since a radioactive metabolite with similar solubility properties was formed in this system even in the absence of drug substrate.

Stimulation of endogenous methylation by imipramine. Soluble fraction equivalent to 10 g of rabbit lung was dialyzed for 20 hr at 5° against isotonic KCl (1·15%).

Aliquots of this preparation were incubated with either S-adenosylmethionine-$^{14}CH_3$ ($7{\cdot}4 \times 10^4$ dpm) and imipramine or S-adenosylmethionine and imipramine-^3H (1×10^6 dpm), as described in Methods. At the end of the incubation, the mixtures were alkalinized by the addition of 1 ml of 0·5 M borate buffer (pH 10). When incubation mixtures containing S-adenosylmethionine-$^{14}CH_3$ and imipramine were extracted

TABLE 3. APPARENT N-METHYLATION OF RELATED COMPOUNDS

Substrate	Radioactivity* (dpm)
DDMI	552
DMI	5418
Imipramine	3212
Nortriptyline	5121

* Radioactivity extracted directly into isoamyl alcohol; values are corrected for endogenous methylation. Soluble fraction equivalent to 25 mg rabbit lung was incubated with substrate (1 μmole), 100 μliters 0·5 M phosphate buffer (pH 7·9), and 7·5 mμmoles S-adenosylmethionine-$^{14}CH_3$. The values are typical of 3 experiments.

TABLE 4. EXTRACTION PROPERTIES OF RADIOACTIVE METABOLITES FORMED IN THE PRESENCE OF IMIPRAMINE AND DMI

Substrate	Radioactivity* extracted	
	Isoamyl alcohol (dpm)	Heptane (dpm)
Imipramine	1368	48
DMI	2268	1847

* Values are corrected for endogenous methylation. Soluble fraction equivalent to 25 mg rabbit lung was incubated and extracted as described in Methods. The values are typical of 3 experiments.

directly with isoamyl alcohol, a large amount of radioactivity was found in the organic solvent. Essentially the same amount of radioactivity was extracted from incubation mixtures into isoamyl alcohol, even after two extractions with heptane to remove unchanged imipramine (Table 5). In contrast, when incubation mixtures containing S-adenosylmethionine and imipramine-^3H were first extracted with heptane, the amount of radioactivity recovered in isoamyl alcohol did not exceed that which was due to imipramine-^3H in unincubated controls. It is thus obvious that imipramine itself is not methylated in this system but that it stimulates the methylation of an endogenous substrate.

Studies with rat lung preparations. As previously reported by Axelrod,[7] the soluble fraction of rat lung was unable to methylate serotonin. Moreover, no methylation of

DMI could be detected in this preparation. Interestingly, when the soluble fraction of rat lung was incubated together with the rabbit lung preparation and S-adenosylmethionine-$^{14}CH_3$, an isoamyl alcohol-extractable product was formed (Table 6). From these results it is reasonable to conclude that rat lung contains a considerable amount of substrate for the transferase enzyme even though the enzyme itself is

TABLE 5. EFFECT OF IMIPRAMINE ON ENDOGENOUS METHYLATION

System	Radioactivity*	
	Control (dpm)	Experimental (dpm)
Imipramine + S-adenosylmethionine-$^{14}CH_3$ ($7{\cdot}4 \times 10^4$ dpm)	205	6354
Imipramine-^3H (1×10^6 dpm) +S-adenosylmethionine	3530	3762

* Radioactivity extracted into isoamyl alcohol after two extractions with heptane. Dialyzed soluble fraction equivalent to 125 mg tissue was incubated with 1 μmole imipramine, 500 μliters 0·5 M phosphate buffer (pH 7·9), and 15 mμmoles S-adenosylmethionine.

In the control experiments, imipramine or imipramine-^3H was added to the vessel at the end of a 60-min incubation and the reaction mixture extracted immediately.

TABLE 6. EFFECT OF THE SUPERNATANT FRACTION OF RAT LUNG

System	Radioactivity* (dpm)
Rat supernatant fraction	111
Rabbit supernatant fraction	157
Rat supernatant fraction + rabbit supernatant fraction	4251

* Radioactivity extracted directly into isoamyl alcohol. Incubation mixtures contained 50,000 g supernatant fraction equivalent to 12·5 mg rabbit lung or 25 mg rat lung, 100 μliters 0·5 M phosphate buffer (pH 7·9) and 7·5 mμmoles S-adenosylmethionine-$^{14}CH_3$. No exogenous substrate was added.

absent. The data, however, do not exclude the possibility that rat lung may contain a substance which stimulates the methylation of an endogenous substrate in rabbit lung.

DISCUSSION

Recent studies have disclosed marked species differences in the relative rates of metabolism of imipramine and DMI.[6, 8] For example, DMI is metabolized more rapidly than imipramine in rabbits, but in rats DMI has a longer half-life than its parent compound. Thus, after the administration of imipramine, DMI accumulates in tissues of rats but not those of rabbits. The species difference in the metabolism of these compounds *in vivo* is reflected by their fate in hepatic microsomes. Rabbit liver microsomes oxidize imipramine mainly to products other than DMI and metabolize

DMI even more rapidly than imipramine itself. In contrast, rat liver microsomes convert imipramine primarily to DMI and transform this metabolite slowly to other products.

The results of the present studies show that the methylation of DMI to imipramine is catalyzed by an enzyme present in the lungs of rabbits but not in those of rats. Since DMI shows a high affinity for lung tissue, this species difference in the methylation of DMI may work in concert with the differences in hepatic metabolism to prevent its accumulation in the tissues of rabbits.

Axelrod[7] has shown that the lung N-methylating enzyme is characterized by a remarkable lack of substrate specificity. The enzyme catalyzes the methylation of a diversity of primary and secondary amines including normally occurring amines, drugs, and other foreign compounds. It is therefore noteworthy that the enzyme methylates DMI more rapidly than DDMI, its primary amine analog.

The administration of imipramine does not affect the levels of endogenous serotonin or norepinephrine in brain.[3] The drug, however, markedly affects the uptake and metabolism of circulating amines. For example, imipramine impairs the uptake of both serotonin and catecholamines in tissues and enhances their rate of metabolism *in vivo*.[9–11] Accordingly, it was suggested that imipramine decreases the uptake and increases the metabolism of the amines by preventing their entry to a protective binding site rather than by causing their release.

The results obtained in the present study indicate that imipramine enhances the methylation of an endogenous substrate in soluble fraction of lung. Previous studies* have shown that imipramine is highly bound by both subcellular fractions and soluble proteins. It is thus suggested that high concentrations of imipramine enhance endogenous methylation by actually displacing an amine substrate from its binding site.

Before a functional role can be suggested for N-methyl transferase in rabbit lung, more information will be required concerning its endogenous substrates. The stimulation of endogenous methylation by imipramine may provide a useful tool in identifying these substrates. It is interesting that preliminary studies indicate that only about 15% of the methylated products formed in the presence of imipramine possess extraction properties similar to those of methylated serotonin analogs.

* J. V. Dingell, "The Physiological Distribution and Enzymatic Metabolism of the Antidepressant Imipramine (Tofranil)," Thesis, Georgetown Univ., Washington, D.C., 1962.

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