In vitro Metabolism of 2-Chloro-10-(3-dimethylaminopropyl)-phenothiazine. III. Isolation and Identification of Metabolites

JOHN J. ROSS, JR., THOMAS L. FLANAGAN, AND ALFRED R. MAASS

Research and Development Division, Smith Kline & French Laboratories,
Philadelphia 1, Pa.

The metabolism of 2-chloro-10-(3-dimethylaminopropyl)-phenothiazine by rat and rabbit liver homogenates was studied. Three metabolites of the -(N-methyl)-C^{14} or S^{35} labeled drug were isolated and identified as $C^{14}O_2$, $HC^{14}HO$ and 2-chloro-10-(3-methylaminopropyl)-phenothiazine-S^{35}.

The effects of various inhibitors upon the oxidation of chlorpromazine-(N-methyl)-C^{14} in the rat liver homogenate system were also investigated.

The demethylation of chlorpromazine (2-chloro-10-[3-dimethyl-aminopropyl]-phenothiazine hydrochloride) has been demonstrated *in vivo* in the rat[1] by showing that the methyl group of chlorpromazine-(N-methyl)-C^{14} was oxidized to carbon dioxide-C^{14} following oral or intravenous administration. These original findings were later extended by the demonstration that chlorpromazine-(N-methyl)-C^{14} was also metabolized *in vitro* to carbon dioxide-C^{14} by rat and rabbit liver homogenates.[2] Walkenstein and Seifter[3] have reported that promazine-S^{35} (10-[3-dimethylaminopropyl]-phenothiazine-S^{35} hydrochloride) is likewise demethylated, by the dog, to the corresponding monomethyl and monomethylsulfoxide derivatives.

The present communication reports the isolation and identification of formaldehyde-C^{14} and 2-chloro-10-(3-methylaminopropyl)-phenothiazine-S^{35} as products of the *in vitro* metabolism of chlorpromazine labeled with the appropriate radioactive isotope. In addition, the ability of several tissues to metabolize chlorpromazine-(N-methyl)-C^{14}, the cofactor requirements for rat liver homogenates and the effect of various metabolic inhibitors on the production of carbon dioxide-C^{14} and formaldehyde-C^{14} are reported.

Experimental

Adult, male Long Evans rats (225–250 g.), maintained on Purina laboratory chow, were sacrificed; the tissue was prepared, fortified and incubated as

(1)   J. J. Ross, Jr., R. L. Young, and A. R. Maass. *Science,* **128,** 1279 (1958).
(2)   R. L. Young, J. J. Ross, Jr., and A. R. Maass. *Nature,* **183,** 1396 (1959).
(3)   S. S. Walkenstein and J. Seifter, *J. Pharmacol. Exptl. Therap.,* **125,** 283 (1959).

previously described[4] except that 0.12 μmole of glucose-6-phosphate was substituted for glucose-1,6-diphosphate.

The formaldehyde-C^{14} present in the mixture at the termination of the incubation was isolated as the dimethyl-dehydroresorcinol derivative (formaldemethone) according to the method of Mackenzie.[5] The formaldemethone-C^{14} was recrystallized to constant specific activity from hot ethanol and counted on a liquid scintillation spectrometer in toluene containing 0.4% of 2,5-diphenyloxazole.

For the isolation and identification of the monomethyl homolog of chlorpromazine, the S^{35}-labeled drug was used instead of chlorpromazine-(N-methyl)-C^{14}. The trichloroacetic acid supernatant obtained after incubation of chlorpromazine-S^{35} with rat liver homogenates in the standard media was adjusted to pH 13 with NaOH and extracted 4 times with diethyl ether. The ether extracts were combined and evaporated to dryness under nitrogen. The residues were taken up in methanol and chromatographed on Whatman No. 1 filter paper in a 3:1:4:2 (v./v.) mixture of ethylene dichloride, benzene, formic acid (88%) and water by the descending technique for 16 hr.[6] The radioactive material at R_f 0.85, which corresponded to the R_f of authentic 2-chloro-10-(3-methylaminopropyl)-phenothiazine, was eluted from the chromatogram with a mixture of absolute methanol and 0.1 N HCl (5:1 v./v.). This eluent was evaporated to dryness under nitrogen, and the residue was dissolved in absolute ethanol. An aliquot of this solution was chromatographed in the ethylene dichloride solvent system concurrently with an aliquot of authentic 2-chloro-10-(3-methylaminopropyl)-phenothiazine which had been eluted from a chromatogram and otherwise treated in the same manner. Both chromatograms yielded one spot, R_f 0.07, which corresponded to authentic 2-chloro-10-(3-methylaminopropyl)-phenothiazine-5-oxide, indicating that the phenothiazine was oxidized to the sulfoxide during the extraction and subsequent chromatography.

Authentic 2-chloro-10-(3-methylaminopropyl)-phenothiazine-5-oxide (100 mg.) was added to the ethanolic HCl extract, obtained as above, next 3 ml. of 1% NaOH solution and 5 ml. of a 3% solution (w./v.) of p-phenylazobenzenesulfonyl chloride in absolute alcohol. The mixture was heated on a steam bath for 30 min. with occasional stirring. An orange, gummy residue formed on the bottom of the reaction flask. The supernatant liquid was discarded; it contained no radioactivity. The residue was dissolved in 25 ml. of hot, absolute ethanol and then allowed to crystallize from this solution overnight at $-6°$. The resulting orange, needle-like crystals were filtered, washed with cold absolute ethanol and dried for 16 hr. in a vacuum desiccator; yield, 47%. A portion was counted in the liquid scintillation spectrometer. The product then was recrystallized to constant specific activity from hot ethanol. The labeled derivative was subjected to infrared spectral analysis, and its spectrum was compared with the infrared spectrum of the p-phenylazobenzenesulfonamido derivative of authentic 2-chloro-10-(3-methylaminopropyl)-phenothiazine-5-oxide.

Discussion and Results

The enzyme system mediating the demethylation of chlorpromazine-(N-methyl)-C^{14} requires the presence of oxygen, glucose-6-

(4) G. C. Mueller and J. A. Miller. *J. Biol. Chem.*, **202**, 579 (1953).

(5) C. G. Mackenzie. *J. Biol. Chem.*, **186**, 351 (1950).

(6) I. B. Eisdorfer and W. C. Ellenbogen. *J. Chromatog.*, **4**, 329 (1960).

TABLE I

METABOLISM OF CHLORPROMAZINE-(N-METHYL)-C^{14} TO $C^{14}O_2$ BY
HOMOGENATES OF VARIOUS RAT TISSUES

Tissue tested in standard incubation mixture	Substrate-C^{14} metabolized to $C^{14}O_2$ mμmole
Liver[a]	3.02
Kidney	0.025
Brain	0.004
Lung	0.295
Boiled rat liver homogenate	0.311
Reagent control[b]	0.005

[a] Contained 4.3 mμmole chlorpromazine-(N-methyl)-C^{14}.   [b] Standard incubation mixture without tissue homogenate.

TABLE II

THE EFFECT OF SUBSTRATE LEVEL ON THE EXTENT OF *in vitro*
METABOLISM OF CHLORPROMAZINE-C^{14} TO $C^{14}O_2$

Mμmole chlorpromazine-C^{14} incubated	Mμmole chlorpromazine-C^{14} metabolized to $C^{14}O_2$
2.2	0.74
4.3	1.79–4.24
22.0	7.34
280	126.3–147.3
363	110.2–124.1
385	123.2
2650	228.2

phosphate (C-6-PO$_4$), diphosphopyridine nucleotide (DPN), triphosphopyridine nucleotide (TPN), nicotinamide, adenosine-triphosphate (ATP), and MgCl$_2$, for optimal activity. In agreement with the demethylation studies of Mueller and Miller[4] only the liver, of the tissues tested, actively demethylated chlorpromazine-(N-methyl)-C^{14} under the conditions of the incubation (Table I). In this experiment the concentration of chlorpromazine-C^{14} in the incubation mixture was one hundredth that in subsequent experiments. However, in the range of 2 to 385 mμmoles of chlorpromazine-(N-methyl)-C^{14}, in a total volume of 2.4 ml., the substrate concentration appeared to have little effect upon the amount of chlorpromazine-C^{14} converted to carbon dixoide-C^{14} (Table II).

At the lower substrate concentrations (2.2 and 4.3 mμmoles), the percentage of the chlorpromazine-(N-methyl)-C^{14} converted to $C^{14}O_2$ varied between 30 and 100. Between 22 and 385 mμmole of chloropromazine-C^{14} per incubation, the recovery of $C^{14}O_2$ from the demethylation of the labeled methyl group fell consistently between 30 to 50% of the incubated drug, but when 2650 mμmoles of sub-

Table III

RECRYSTALLIZATION OF THE DIMETHYLDEHYDRORESORCINOL DERIVATIVE
OF FORMALDEHYDE-C^{14} TO CONSTANT SPECIFIC ACTIVITY

Crystalli-zation	Rat liver homogenate		Rabbit liver homogenates	
	Total act., cpm.	Specific radioactivity, cpm./mg.	Total act., cpm.	Specific radioactivity, cpm./mg.
1	5179	117.7	20043	260.3
2	1246	18.5	8723	147.1
3	528	20.7	5338	116.8
4	490	20.6	4037	117.7

strate were incubated under standard conditions the extent of demethylation as measured by $C^{14}O_2$ liberation was only 8%. The data would seem to indicate that the optimum substrate level for maximum demethylation of chlorpromazine-(N-methyl)-C^{14} to $C^{14}O_2$ lay in the range of 280 mμmole of the drug. Further studies were not performed to establish this point more precisely.

The several reports in the literature that formaldehyde is an intermediate in the conversion of the methyl group of aliphatic mono- and dimethylamines to carbon dioxide by rabbit, rat and guinea pig liver homogenates[4,5,7] prompted us to search for formaldehyde-C^{14} as a metabolite of chlorpromazine-(N-methyl)-C^{14} by rat and rabbit liver homogenates. The formaldehyde-C^{14} was isolated as the dimethyldehydroresorcinol derivative and recrystallized to a constant specific activity (Table III).

β-Diethylaminoethyl diphenylpropylacetate hydrochloride (SK&F 525-A) which has been reported to inhibit amine demethylation,[8] inhibited the conversion of chlorpromazine-(N-methyl)-C^{14} to $C^{14}O_2$ and also increased the amount of $HC^{14}HO$ isolated from the rat and rabbit liver homogenates (Table IV). Aminopterin, which is a potent competitive inhibitor of 1 carbon transfer reactions, also markedly reduced the metabolism of chlorpromazine-C^{14} to $C^{14}O_2$, suggesting that formyl- or hydroxymethyl-tetrahydrofolic acid may be involved in the demethylation procedure.[8]

Following the suggestion of Mueller and Miller[4] that sulfhydryl groups may be involved in the demethylation reaction, it was found that glutathione and cysteine also reduced the formation of $C^{14}O_2$. Glutathione, 6.8×10^{-3} molar, almost completely inhibited the production of $C^{14}O_2$ when incubated in the standard rat liver homogenate with chlorpromazine-(N-methyl)-C^{14}. However, the accumulation of $HC^{14}HO$ was increased four-fold over the control incubation

(7) B. N. La Du, L. Gaudette, N. Trousof, and B. B. Brodie, *J. Biol. Chem.*, **214**, 741 (1955).

(8) F. M. Huennekens, M. J. Osborn, and H. R. Whitley, *Science*, **128**, 120 (1958).

TABLE IV

THE EFFECT OF INHIBITORS ON THE *in vitro* METABOLISM OF CHLORPROMAZINE

Species	Inhibitor	Molar concentration of inhibitor	Substrate-C^{14} metabolized to $C^{14}O_2$ mμmole	Substrate-C^{14} metabolized to $HC^{14}HO$ mμmole
Rat[a]	SK&F 525-A	None	118.5	0.6
		1×10^{-2}	0.7	6.8
		1×10^{-3}	0.8	3.4
		2×10^{-4}	2.8	2.6
Rabbit[b]	SK&F 525-A	None	130.1	4.9
		1×10^{-2}	99.8	6.9
Rat[a]	Aminopterin	None	118.5	0.6
		1×10^{-7}	1.5	4.1
Rabbit[b]	Aminopterin	None	130.1	4.9
		1×10^{-7}	1.2	13.8
Rat[a]	Control	None	118.5	0.6
	Glutathione	6.8×10^{-3}	0.003	2.6
	Cysteine	6.7×10^{-3}	0.2	7.3

[a] Substrate concentration was 363 mμmole. [b] Substrate concentration was 294 mμmole.

(Table IV). Cysteine at levels equivalent to that of glutathione (6.7×10^{-3} molar) also markedly inhibited the production of $C^{14}O_2$ and increased the formation of $HC^{14}HO$ (Table IV). Mueller and Miller[4] reported this type of phenomenon when 3-methyl-4-mono-ethylaminoazobenzene was incubated under these conditions. They postulate that glutathione effectively trapped a hydroxymethyl intermediate as a mercaptal derivative during the oxidation of the N-methyl group. The present data indicate that the mechanism for the inhibition of demethylation of 3-methyl-4-monoethylamino-azobenzene by glutathione may also apply to the demethylation of chlorpromazine-C^{14}.

The initial experiments to isolate the primary or secondary amine metabolites of chlorpromazine using the N-methyl-C^{14} form of the drug were unsuccessful. The loss of the C^{14} label during the incubation added to the difficulties of the isolation. Therefore, chlorprom-azine-S^{35} was incubated with standard rat liver homogenates and the S^{35}-labeled metabolites of the drug were extracted from the incubation mixture. 2-Chloro-10-(3-methylaminopropyl)-phenothiazine-S^{35} was isolated and tentatively identified by chromatography (Figure 1). The radioactive spot corresponding to 2-chloro-10-(3-methylamino-propyl)-phenothiazine-S^{35} was eluted from the chromatogram, and treated with *p*-toluenesulfonyl chloride to form the amido derivative. All attempts to form this derivative with material eluted from the
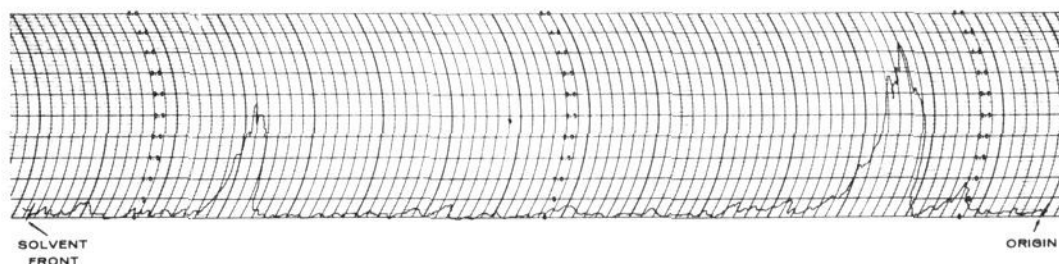
Fig. 1.—Paper chromatogram of S^{35}-labeled metabolite of chlorpromazine-S^{35} extracted from standard rat liver homogenate incubation. The R_f of the peaks in order from the origin are, 2-chloro-10-(3-methylaminopropyl)-phenothiazine-5-oxide, 2-chloro-10-(3-dimethylaminopropyl)-phenothiazine-5-oxide, and 2-chloro-10-(3-methylaminopropyl)-phenothiazine and these correspond to peaks obtained with authentic samples of these same compounds.
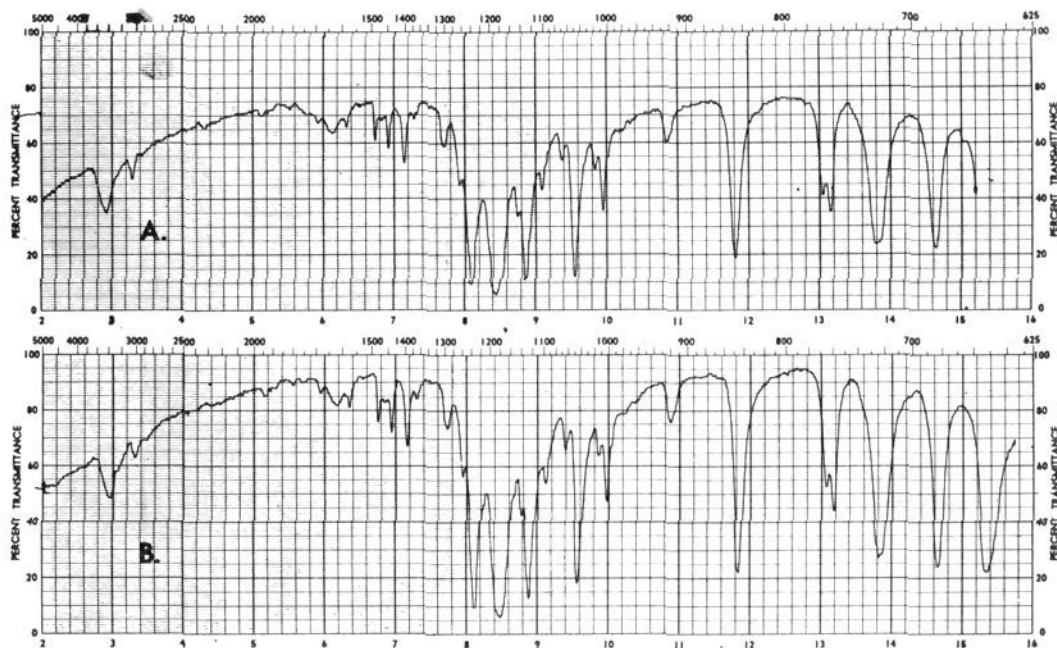

Fig. 2.—Infrared spectra of the p-phenylazobenzenesulfonamido derivatives of 2-chloro-10-(3-methylaminopropyl)-phenothiazine-5-oxide. Spectrum A was obtained from the derivative prepared from authentic 2-chloro-10-(3-methylaminopropyl)-phenothiazine-5-oxide. Spectrum B was obtained from the derivative prepared from the oxidized S^{35}-labeled metabolite isolated from the standard rat liver homogenate incubation of chlorpromazine-S^{35} by extraction and paper chromatography.

chromatogram failed. It was then found that small quantities of authentic 2-chloro-10-(3-methylaminopropyl)-phenothiazine, when eluted from a chromatogram with acidic methanol, were oxidized to 2-chloro-10-(3-methylaminopropyl)-phenothiazine-5-oxide, which did not undergo the p-toluenesulfonyl chloride reaction to form

TABLE V

RECRYSTALLIZATION OF THE *p*-PHENYLAZOBENZENESULFONYLAMIDE TO
2-CHLORO-10-(3-METHYLAMINOPROPYL)-PHENOTHIAZINE-5-OXIDE-S[35] TO
CONSTANT SPECIFIC ACTIVITY

Crystallization	Total activity, cpm.	Specific radioactivity cpm./mg.
1	731	9.2
2	630	9.0
3	556	9.0

the desired derivative; however the oxide did react to form the *p*-phenylazobenzenesulfonamido derivative. This derivative was therefore recrystallized to constant specific activity (Table V) and identified by infrared spectroscopy.

The infrared spectrum of the *p*-phenylazobenzenesulfonamide derivative prepared from authentic 2-chloro-10-(3-methylaminopropyl)-phenothiazine and that obtained in these experiments were identical (Fig. 2).