have greater influence on esterification of cholesterol.

Whether gammexane brings about inhibition of cholesterase, the enzyme system responsible for esterification of cholesterol in liver(17) and pancreas(18), in a manner reversible by excess inositol is under investigation.

Lowering of total cholesterol and increase of ester fraction has been observed by Avigan and Steinberg(19) by feeding rats with unsaturated fatty acids. Klein(20) demonstrated that serum cholesterol is preferentially esterified with poly-unsaturated fatty acids. Therefore the possible role of inositol directly or indirectly influencing the liver to bring about unsaturation of fatty acid deserves further study.

Summary. Tissues of *Corcyra cephalonica* and serum of albino rats fed a diet containing gammexane accumulated more free cholesterol with concomitant decrease in ester fraction. Inositol almost completely counteracted the effect of gammexane. Inositol thus appears to have great influence on esterification of cholesterol.

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Metabolism of Chlorpromazine: Organic-Extractable Fraction From Human Urine.* (25741)

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Despite its widespread clinical use, no detailed data are available on the metabolism of chlorpromazine in humans. Experiments with dogs have led to isolation of chlorpromazine sulfoxide from urine(1) and to renewed emphasis on sulfoxidation as a major metabolic pathway common to the phenothiazines(1,2, 3). More recently, Walkenstein and Seifter (3) have demonstrated that the side chain of promazine (and chlorpromazine) undergoes N-monodemethylation in animals. This finding would probably account for the release of $C^{14}O_2$ following administration of chlorpromazine-(N-methyl)-C^{14} to rats(4).

The present work was undertaken preparatory to a more general study on biochemical and therapeutic significance of variations in

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chlorpromazine metabolism in psychiatric disorders. As indicated below, improved chromatographic methods have enabled the authors to demonstrate 2 new major metabolites in organic extracts of human urine, in addition to other phenothiazine derivatives. The urinary excretion patterns obtained appear to differ significantly from observations based on animal studies in other laboratories.

Experimental. Urine specimens. These were collected from 40 male psychiatric patients at the Creedmoor State Hospital who were receiving 300-600 mg of chlorpromazine orally per day. To eliminate specimens from noncooperative patients, each urine was first screened with Subetest reagent (Subet Laboratories, New York), which was employed to confirm chlorpromazine intake. Control urines were provided by laboratory personnel who were not on medication.

Chromatographic work-up. Urine (100 ml) was mixed with 4 ml of 3 N NaOH and shaken mechanically with ethylene dichloride (120 ml), which proved to be superior to ether as an extractant.† The organic extract was washed with 0.1 N NaOH (60 ml), dried over sodium sulfate and the volume measured before being taken to dryness. The residue was redissolved in 0.003 of its original volume of ethylene dichloride and appropriate aliquots (5 to 50 lambdas) were spotted on Whatman #1 sheets marked "for chromatography." The following 3 chromatographic systems were used: (a) Benzene-acetic acid-water (2:2:1), descending, 21-22°C (temperature critical). For optimal results, the jar (rectangular, provided with paper curtain) was equilibrated over the week-end with both the aqueous phase (below) and organic phase (in trough). A "dry run" was then made with a blank sheet of paper before the spotted sheets were hung from the troughs, using a fresh change of organic phase. After terminating the run, it was possible to re-use the same jar for successive chromatographic separations. (b) Ethylene dichloride-benzene-formic acid (88%)-water (3:1:4:2),‡ descending, 20-22°. The spotted sheets were equilibrated overnight with the aqueous phase. (c) Butanol-ammonium acetate (1 M, pH 7) (2:1), descending. Chromatograms were routinely dipped in Dragendorff-ethyl acetate reagent(5) to visualize the various chlorpromazine metabolites. The following color reagents were also employed: ninhydrin-pyridine dip (ref. 6, p. 66), for detecting primary amines; nitroprusside-acetaldehyde dip(3), for secondary amines; acidic iron dip, prepared from 1 vol. methanol plus 1 vol. $FeCl_3 \cdot 6H_2O$ (1% in 2.5 N HNO_3); and persulfate dip (ref. 6, p. 125). For the spectrophotometric studies, the individual components were eluted from unstained duplicate chromatograms with 0.1 N HCl and read in the Beckman DU spectrophotometer. Methanol was employed for elution when the individual spots were to be rechromatographed. The various standards employed for reference purposes in this study were obtained from the Smith Kline and French Laboratories through the courtesy of Dr. Van Loon.

Results.§ Preliminary studies carried out in our laboratory(7) indicated that organic extracts of urine voided by subjects on CP therapy could be resolved into 3 fractions by paper strip electrophoresis using 1% $KHCO_3$ as the solvent. The first fraction remained at point of application, similar to the behavior of CP. A second fraction showed moderate movement towards the cathode and had a principal UV absorption peak at 255 mμ. The third fraction migrated rapidly to the cathode; in this respect, as well as in its UV

† In our more recent experiments, urine is extracted a second time to remove additional amounts of metabolic material—particularly compound #4, which is voided in large quantities (cf. *Results*).

‡ Separation of organic and aqueous phases is facilitated by permitting the mixture to sit overnight in a separatory funnel. Note that the phases have almost identical specific gravities and may invert with small changes in temperature.

§ The following abbreviations are used: BzA = benzene-acetic acid-water; EdBzF = ethylene dichloride-benzene-formic acid-water; BuAmA = butanol-ammonium acetate; CP = chlorpromazine = 10-(3′-dimethylaminopropyl)-2-chlorophenothiazine; Nor_1CP = desmethylchlorpromazine; Nor_2CP = desdimethylchlorpromazine. Chlorpromazine sulfoxide (CPSO) and its sulfone ($CPSO_2$) are assigned similar prefixes (Nor_1 or Nor_2) to designate desmethyl derivatives.

TABLE I. Comparison between Urinary (Nonpolar) Chlorpromazine Metabolites and Several Authentic Chlorpromazine Derivatives.

Substance	R_f (BzA)	Standards with identical R_f's and color reactions*	Color reactions† N	N-a	I	P
Urine						
1	.07		—	—	—	±
2	.16	Nor_2CPSO	++	—	—	+
3	.25(0.24)‡	Nor_1CPSO	±	+	—	+
3′	.25(0.27)‡	CPSO	—	—	—	+
9	.29				—	+
4	.33		—	—	—	+
8	.37		—		—	+
5	.64		—	—	+	+
6	.74	CP	—	—	+	+
7	.87		—	—	?	+
Misc. st'ds						
$CPSO_2$	.40		—	—	—	—
Nor_2CP	.67		++	—	+	+
	.87		—	—	?	+
Nor_1CP	.74		±	+	+	+

* All reference standards were treated like urine, *i.e.*, extracted with ethylene dichloride from aqueous alkaline solution. R_f's obtained in this manner were slightly greater than when standards were spotted directly. Nor_2CPSO, Nor_1CPSO and CPSO were compared with corresponding urine spots in 3 solvent systems, *viz.*, BzA, EdBzF and BuAmA; CP was tested in one system (BzA).

† N = ninhydrin; N-a = nitroprusside-acetaldehyde; I = iron; P = persulfate.

‡ R_f's given in parentheses refer to standards in third column. Color reactions of spots #3 and #3′ were determined after separation with EdBzF.

spectrum, it corresponded to CPSO. However, analytical tests revealed the presence of at least 2 phenothiazine derivatives in the first and third fractions. Further separation via electrophoresis could not be obtained by varying the buffer pH or other parameters. Alternative attempts at a complete fractionation by paper chromatography using butanol-acetic acid-water and BuAmA were also unsuccessful.

A more effective resolution of the urine extracts was obtained upon introduction of the BzA system. Paper chromatography, under the conditions outlined in *Experimental,* yielded 7 discrete spots (#1 to #7, Table I) with both the Dragendorff and persulfate reagents. These substances were shown to be related to chlorpromazine since they did not appear in the normal controls. Furthermore, the first, second and third spots yielded UV absorption maxima similar to CPSO and its desmethyl derivatives, *viz.*, 240, 275, 300 and 340 mμ, in contrast to the spectra obtained from normal urinary chromatograms.

When the third spot was eluted with methanol and rechromatographed with EdBzF or BuAmA, 2 components were noted, designated #3 and #3′. EdBzF proved to be especially useful for separating the sulfoxide metabolites. With this system (as with BzA), CP and related non-sulfoxides were as a rule carried along more rapidly than the sulfoxides. However, the bulk of the Dragendorff-positive material remained behind close to the starting point. By allowing the solvent to run off the paper for a period of 24-48 hours the slow-moving compounds could be effectively separated. This procedure revealed 2 new minor metabolites, #8 and #9. These metabolites flank spot #4 in the BzA chromatogram (Table I) but were not previously detected because of their proximity to this major metabolite. The spots obtained with EdBzF have the following order of increasing R_f's: (#2, #4) < #9 < #3 < #3′ < #8 < (#5, #6).

Ultraviolet studies revealed that 6 compounds, *viz.* #1, #2, #3, #3′, #8 and #9 exhibit the sulfoxide spectrum. Compounds #4 through #7 are apparently not sulfoxides, but their absorption spectra could not be accurately plotted due to background interference from aromatic material normally present in urine. No evidence was found for $CPSO_2$, whose UV absorption maxima (235, 270, 295 and 335 mμ), R_f and persulfate reaction failed to correlate with any of the observed metabolites (Table I).

The ninhydrin and nitroprusside reagents were used to determine whether demethylation had occurred. One compound (#2) reacted as a primary amine and one compound (#3) reacted as a secondary amine. From their R_f's and color reactions the following tentative assignments have been made: #2 = Nor_2CPSO, #3 = Nor_1CPSO, #3′ = CPSO and #6 = CP (cf. Table I). Definitive evidence for presence of Nor_1CP and Nor_2CP on urinary chromatograms could not be found. However, it should be noted that Nor_2CP, as judged by the behavior of the standard supplied to us, was either impure or

unstable in aqueous solution and yielded 2 spots on the chromatogram (Table I). The second, anomalous spot was ninhydrin-negative and apparently an impurity, but it was of interest that its R_f (0.87) and color reactions matched urine compound #7. The significance of these observations and their relevance to origin of compound #7 is now being studied.

Discussion. The findings reported above are not in complete harmony with current concepts concerning the metabolism of chlorpromazine. Although CPSO may serve as an important metabolic intermediate, it constitutes but one (minor) component among the phenothiazine derivatives which may be detected in organic extracts of human urine. Compounds #2, #3 and #4 appear from our studies to be the major extractable products of transformation. Compound #3 (Nor_1-CPSO) was reported earlier in urine of dogs (3). However, the Nor_2sulfoxide (#2) and compound #4 are presented here for the first time. (See *Addendum*)

Though there have been previous indications that monodemethylation (of promazine) occurs in animals to yield Nor_1promazine as well as its sulfoxide, complete demethylation to form the Nor_2 derivatives has been denied (3). In our work with humans we have been unable to demonstrate Nor_1CP, whereas Nor_2CPSO was readily distinguished. The observed discrepancies may be due to difference in species. However, it is noteworthy that Nor_2CPSO is not readily extracted by ether—which is the solvent employed by other workers—and this may account for earlier failures to record this major metabolite.

In addition to the compounds present in organic extracts of alkaline urine, numerous metabolites may be demonstrated in the residual urine(8). These polar derivatives are readily adsorbed on Amberlite CG-50 carboxylic acid resin and are responsible for the purple colors noted when urine is treated with acid containing ferric or nitrite ions. Several compounds present in this second fraction are phenolic derivatives which are linked to glucuronic acid (cf. ref. 9). It therefore appears that the pattern of chlorpromazine metabolism is not quite so simple as previously considered, but involves sulfoxidation, demethylation, ring hydroxylation and other as yet undetermined chemical changes.

During the present study, patients on chlorpromazine therapy tended to differ considerably in their metabolite patterns. Both qualitative and quantitative variations were observed. In an unusual case, urine extracts which gave characteristic sulfoxide UV absorption spectra were later shown to be almost devoid of CPSO and its demethylated products (spots #2, #3 and #3′), in contrast to the relatively abundant substances #1 and #4. Whether this variation is correlated with drug dosage, diet, clinical response to the drug, or psychiatric disorder, or reflects individual differences, has not been determined. Further studies along these lines are indicated.

Summary. Chromatographic studies reveal the presence of 10 Dragendorff (+) compounds in organic (ethylene dichloride) extracts of alkalinized human urine following administration of chlorpromazine. Six of these compounds appear to be sulfoxides. Included in this group are chlorpromazine sulfoxide—which is a relatively minor product—as well as 2 major metabolites whose chromatographic behavior is indistinguishable from Nor_1 and Nor_2chlorpromazine sulfoxide. A third major metabolite (compound #4) remains unidentified, but it is not a sulfoxide derivative. It is indicated that the residual urine remaining after extraction contains numerous additional (polar) metabolites.

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Addendum. Present studies suggest that compond #4 may not be a phenothiazine derivative. This conclusion is based on two observations: (1) The compound has only been observed in a small proportion of patients at the Hillside Hospital, in contrast to the Creedmoor Hospital group; (2) Following its elution from the BzA chromatogram, purification, and

rechromatography in the EdBzf system, #4 appears to lose its persulfate-staining properties. This observed change in staining behaviour has not been noted for the other compounds tested (#2 through #6). There is tentative evidence that the presence of compound #4 in urine may be related to the use of tobacco.

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Effects of Tetrazotized Diorthoanisidine on Depressor Response to 5-Hydroxytryptamine in the Rabbit. (25742)

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Diazo and tetrazo salts react *in vitro* with 5-hydroxytryptamine (5-HT) to form blue colored azo dyes(1). If similar reactions can occur *in vivo* these salts should block the pharmacological actions of 5-HT unless the coupled reaction by-product retains 5-HT activity. Results of previous studies indicate that the tetrazo salt, tetrazotized diorthoanisidine (TDA) is an adrenergic blocking drug (2). The blue colored complex formed by reacting TDA with 5-HT does not possess 5-HT activity on blood pressure of dogs or rabbits even in doses equivalent to 200 μg of 5-HT. We, therefore, decided to determine whether TDA could block some of the effects of exogenously administered 5-HT. Rabbits were used in all experiments since 5-HT produces a hypotensive response in this species. Other workers have shown that sympatholytic agents can modify the pressor effects of 5-HT but do not significantly alter its depressor action in the rabbit(3).

Methods. Albino rabbits weighing 2-3 kg were used. The technic of Horita(4) utilizing the pyretogenic effects of 5-hydroxytryptophane in animals pretreated with iproniazid was used as an index of central effects of 5-HT. Rectal temperatures were recorded at various time intervals in unanesthetized control animals and in others pretreated with TDA. Blood pressure responses to 5-HT, l-epinephrine and l-norepinephrine were recorded continuously from a carotid artery of atropinized, vagotomized rabbits before and after treatment with TDA. These animals were anesthetized with pentobarbital (25-30 mg/kg). Supplementary doses were given as needed.

Results. Pyretogenic action of 5-HT. Iproniazid was administered subcutaneously in 50 or 100 mg/kg doses. Two to 3 hours later 20 mg/kg of 5-hydroxytryptophane were injected intravenously. Rectal temperatures of rabbits so treated were found to be increased markedly within one hour. According to Horita and Gogerty(4) this response is the resultant action of 5-HT accumulation in brain tissue brought about by monoamine oxidase inhibition with iproniazid. A total of 14 rabbits received iproniazid and 5-hydroxytryptophane treatment. Seven of them also were injected intravenously with 20 mg per kg doses of TDA immediately before or following 5-hydroxytryptophane administration. No apparent differences in rectal temperature

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