in upper unit 2 (previously refrigerated) were opened and the foil-capped free end of the tubing was threaded through the clamps and through a small opening which guided it from the bottom of unit 2 into the top of unit 1. With aseptic precautions the aluminum foil cap was removed and a 35 cm long piece of silicone rubber tubing was attached. This bore at its free end a short length of 7 mm glass tubing surrounded at its upper portion by a 2 cm length of silicone tubing. This fitted snugly into arm D of the culture flask, replacing the vaccine stopper, and completed the connection from the reservoir tube to the flask. The clamps in unit 2 were now shut and the screw clamps at the base of the reservoir tubes were opened. The refrigerator was set to change to the heater at 9:15 p.m. and the change of medium was set for 10 p.m. The following morning each connecting tube was removed and replaced with a vaccine stopper in arm D, sterile precautions being observed throughout.

Since the flasks so far used have a lower compartment (B, Fig. 1) able to hold fluid from only 2 changes of medium, this compartment was emptied once each day. A sterile needle was inserted through the vaccine stopper into arm E and the fluid drawn off with a syringe.

Discussion. With the same medium used previously (Trager(2)), extracellular survival and development of *P. lophurae* have been as good in the automatic changer as in experiments in which the medium was changed by pipetting. On day 4 about 80% of the parasites appeared in good connection by morphological criteria. Recently, a modification of the medium has given good survival in the automatic changer to day 5, with infective parasites demonstrated on day 6.

The apparatus here described is not designed for the same purpose as apparatus providing a continuous slow flow of medium. Apparatus of the latter type would not be suitable since the medium used at present for extracellular maintenance of malaria parasites contains labile ingredients, such as adenosinetriphosphate and, in addition, the concentrated red cell extract contains hydrolytic enzymes. Medium held warm in a reservoir providing a slow flow would deteriorate as much as the medium in the culture vessel. With the present method of sudden change, however, the fresh medium can be refrigerated until shortly before it is added to the culture flask. It then provides at the outset high concentrations of labile factors, which presumably are much reduced by the end of the 12-hour period between changes of medium.

Summary. A special flask and mechanical equipment are described which provide for automatic removal at a desired time of old culture fluid and addition of fresh medium to cultures in which extracellular malaria parasites (*P. lophurae*) are attached as a scum to a thin plasma clot lining the wall of the flask.

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Species Dependence of Chlorpromazine Metabolism.* (26884)

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Earlier reports from this laboratory(1,2) have confirmed the importance of sulfoxidation as a pathway for the metabolism of chlorpromazine(3,4). However, the question

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has been raised whether chlorpromazine sulfoxide (CPSO)† itself is a significant urinary excretion product(1,2). Detailed chromatographic studies(1,2) on human urine collected after oral chlorpromazine therapy revealed only minor amounts of CPSO; in contrast, 2 major unconjugated metabolites were detected whose properties were indistinguishable from the desmethyl sulfoxides (Nor_2 CPSO and Nor_1CPSO). These findings were somewhat unexpected, since CPSO has achieved much prominence in the literature (3,4,5), whereas the nor_2sulfoxide had not been previously reported.

The purpose of the present work was 2-fold: (a) To provide additional confirmatory evidence for the desmethyl sulfoxides; and (b) To determine the basis for the divergent findings between our group and other workers. Towards this end, a comparison has been made between chlorpromazine metabolism in man and animals. The dog was selected for the study because much of the literature is based on this animal without sufficient evidence that the findings are applicable to humans.

Experimental. Urine specimens. Twenty-four hour urine collections were contributed by Hillside Hospital patients of both sexes who were receiving 600 mg chlorpromazine hydrochloride orally per day. Creatinine determinations as well as replicate analyses were carried out to assure true 24-hour specimens. For the animal studies, 4 female mongrel dogs received 8 mg of chlorpromazine hydrochloride/kg/day in oral form. The urine was collected daily for 5 days and kept under refrigeration to minimize decomposition. Control urines were obtained from patients and animals who were not on medication.

Chromatography. The urinary chlorpromazine metabolites were resolved into a non-polar fraction (*A*) and 2 polar (phenolic) fractions, designated *B* and *C*. Fraction *A* was extracted from urine at pH 11.5 with 3 volumes of ethylene dichloride‡ and prepared for chromatography essentially as described previously(1). The residual urine was extracted at pH 9 (Fraction *B*) and again at pH 9 (Fraction *C*) after it had been incubated overnight with one-tenth volume of β-glucuronidase (ketodase, 5,000 units/ml) at pH 4.5. *B* and *C* were chromatographed with the following solvent systems: BzA, EdBzF, n-butanol-ethanol-water (15:2:5) and ammonia (58%)-methanol (1:1). The last system employed Whatman #3 paper that had been previously dipped in acetone containing 10% peanut oil.

Quantitative Analyses. Three methods were used to quantitate the chromatograms, the particular choice being dependent on the relative amounts of metabolites present as well as their proximity to each other. Trace metabolites were stained with persulfate reagent and promptly estimated by visual comparison with a series of graded standards. Derivatives present in substantial or major amounts were stained with Dragendorff reagent and quantitated by means of a recording densitometer (Spinco Analytrol Model RB, B-5 cam) at 500 mμ, slit width 4 mm. When the chromatographic spots were sufficiently discrete from one another, it was possible to employ an alternative procedure as follows. The corresponding areas on unstained duplicate chromatograms were cut out, then eluted by shaking mechanically with 2 portions of methanol. The eluates were filtered and taken to dryness. To the residues were added 0.1 ml water, 0.2 ml of 0.5% methyl orange, 0.2 ml saturated boric acid and 5 ml benzene-isoamyl alcohol (100:5, v/v). The tubes were shaken, centrifuged, and the upper organic extracts containing the dye-amine complexes were read *vs.* a reagent blank at 430 mμ. Metabolite readings were corrected by deducting the significant absorbances given by control urine specimens which were chromatographed along with the test urine samples.

† The following abbreviations are used: CP = chlorpromazine, CPSO = chlorpromazine sulfoxide, nor_1 = desmonomethyl, nor_2 = desdimethyl, P = promazine, BzA = benzene-acetic acid-water (2:2:1), EdBzF = ethylene dichloride-benzene-formic acid-water (3:1:4:2), and BuAmA = butanol-ammonium acetate (1 M, pH 7)(2:1).

‡ Eastman reagent grade used. Spurious results were obtained with 2 other commercial preparations. (This could be prevented by redistilling to remove a yellow-colored residue).

TABLE I. Chromatographic Evidence for Nor_2-CPSO and Nor_1CPSO Excretion in Human Urine.

	R_f before and after acetylation			
	BzA		BuAmA	
Compound	Before	After	Before	After
Nor_2CPSO	.17	.77	.68	.82
#2	.16	.77	.68	.81
Nor_1CPSO	.24	.79	.76	
#3	.25	.78	.76	

Small corrections were also applied to the standards, based on the readings obtained by eluting the appropriate areas on "blank" chromatograms.

Acetylation Technic. Urine metabolites #2 and #3 were isolated (elution with methanol) from replicate chromatograms in sufficient amount to yield approximately 0.5 mg of each compound. The solutions were taken to dryness and the residues redissolved in 1 ml of 0.01 N HCl. Nor_2CPSO and Nor_1CPSO served as reference standards. They were dissolved as their hydrochlorides in 1 ml water. The solutions were chilled. To each were added 0.1 ml acetic anhydride and 0.2 g sodium bicarbonate with stirring. All mixtures were clear after 25 minutes. The solutions were mixed with 20 ml ethylene dichloride and 0.2 ml cold 1 N NaOH, then shaken and centrifuged. The organic extracts were washed with a small amount of water and dried over sodium sulfate. They were then reduced in volume to 0.5 ml for chromatographic evaluation.

Results. Evidence for Nor_2CPSO and Nor_1CPSO. Reference was made earlier(1) to 2 major metabolites of chlorpromazine (#2 and #3) which are resolved by prolonged chromatography in EdBzF. From their UV spectra, color reactions and chromatographic behavior it was suggested that these compounds are the desmethyl sulfoxides, Nor_2CPSO and Nor_1CPSO. Additional confirmatory evidence is presented in Table I. The indicated compounds were isolated from urine and acetylated as described in *Experimental,* above. The resultant acetyl derivatives were chromatographed in the BzA and BuAmA solvent systems. Their R_f's proved identical to authentic acetyl Nor_2CPSO and acetyl Nor_1CPSO, respectively. These data supplement our observations that compound #2 = Nor_2CPSO and #3 = Nor_1CPSO as adduced by co-chromatography in 3 systems, *viz.,* EdBzF, BzA and BuAmA.

Species Studies. A comparison between man and dog with respect to their metabolism of chlorpromazine revealed some similarities as well as striking differences. Following their placement on oral chlorpromazine medication, dogs void 9 phenothiazine derivatives in Fraction *A* which are chromatographically similar to the 9 compounds previously detected in human urine (ref. 1, Table I).§ Four of these compounds have been identified (#2, #3, #3′ and #6). In addition, dogs excrete trace amounts of a tenth compound (designated #6′) in Fraction *A* which has not been found in significant quantities in human urine. This derivative was detected by eluting spot #6 from the BzA chromatogram with methanol and rechromatographing it with ammonia-methanol (1:1). Two spots now appeared, the major one at R_f 0.03 (chlorpromazine) and a minor one at R_f 0.13. The minor constituent reacted as a secondary amine (positive nitroprusside, weak ninhydrin) and was chromatographically identical to Nor_1CP. There was no evidence for Nor_2CP, whose R_f in this solvent system is 0.25.

Fraction *A* metabolites #1, #5, #7, #8 and #9 have not been identified. With the exception of #5, these compounds occur in minor amounts in pH 11.5 extracts. However, there is some question whether #1 and #9 are actually minor metabolites. They differ from the other Fraction *A* components by yielding a lavender, rather than pink, persulfate test, and correspond in R_f to 2 components of Fraction *B*. This evidence suggests that they are present in *A* as "contaminants." Further

§ Reference was made in this earlier paper to a tenth compound (compound #4) which appeared as an artifact in the urine of smokers. This material has been identified as cotinine, a metabolite of nicotine(6). Nicotine itself also appears in lesser amount in the urine of smokers at an R_f comparable to metabolite #1, but neither nicotine nor cotinine yields a positive persulfate test. As a consequence, they can be differentiated from the phenothiazine derivatives.

TABLE II. Quantitative Findings by Various Workers (Expressed as Average % of Administered Drug).

	Chlorpromazine				Promazine
	Present workers*		Salzman and Brodie		Walkenstein and Seifter
Urinary product	(man)†	(dog)	(man)	(dog)	(dog)
Nor_2sulfoxide	3.7 ± .6	1.1 ± .1			
Nor_1sulfoxide	1.8 ± .5	5.1 ± .8			3
Sulfoxide	.4 ± .08	5.1 ± .7	5	12	4
CP or P	.2 ± .06	2.8 ± .6	—	1.3	2.5
Subtotal	6.1	14.1			
Probable total, Fraction *A*	7.5	17.			

* Values represent mean ± stand. dev.
† Male patients, avg wt 75 kg. Analyses based on four 24-hr urine specimens.

consideration of these compounds will be given in a subsequent paper. Compound #5 (mentioned above) appears to be a sulfide whose UV spectrum is similar to chlorpromazine. Its color reactions provide no evidence for demethylation or for the presence of a nuclear oxygen atom. We suspect that the compound is chlorpromazine-N-oxide, but definitive proof of this is not presently available.

Quantitative excretion values for CP, CPSO, Nor_1CPSO and Nor_2CPSO are summarized in Table II. It is clear that in neither man nor dog is CPSO the predominant sulfoxide. In fact, CPSO is a *minor* metabolite in man and is voided to the extent of less than 0.5% of the administered CP. The major human sulfoxide is the desdimethyl derivative, Nor_2CPSO (3.7%), with the monomethyl compound (Nor_1CPSO) following in the order of 1.8%. CP appears in trace quantities, 0.2%. Dogs excrete substantial and equal amounts of CPSO and Nor_1CPSO (5.1%), while their output of Nor_2CPSO is only one-fifth as great but still exceeds the CPSO excretion in man. They void, on the average, more unaltered CP than man. These results indicate a species difference for the nonpolar fractions of man and dog with respect to the sulfoxides as well as the unaltered CP.

Man excretes less than one-half as much Fraction *A* material as the dog (Table II), but he compensates for this by producing a greater amount and variety of phenolic metabolites (Fractions *B* and *C*). The metabolic patterns are complex and cannot be considered here in detail. However, it is pertinent to note that there is a *striking qualitative difference* between the 2 patterns. Human urine contains 2 series of phenolic derivatives, one reacting blue and the other lavender to the persulfate reagent. *The blue series appears to be entirely absent from dog urine.* The relative poverty of phenolic material in the urine of dogs receiving CP medication probably accounts for our observation that such urine gives a weak test with the ferric chloride reagent(7), in contrast to urine from humans on an equivalent drug regimen.

Discussion. Our animal excretion values for CP, CPSO and Nor_1CPSO can be compared in Table II with the values for the analogous members of the promazine family which were reported by Walkenstein and Seifter. Good agreement is noted despite the many differences in experimental procedure between the 2 laboratories. The Salzman and Brodie data are also in reasonable agreement with respect to the excretion of CP by man and dog, but differ as to the CPSO output. These authors based their assay on a method (UV spectrophotometry) which also reflects other sulfoxides present in their extracts. This interpretation is borne out by comparing their "CPSO" values to total sulfoxide output (CPSO + Nor_1CPSO + Nor_2CPSO) calculated from our own data. The totals obtained for man (5.9%) and dog (11.3%) agree quite well with Salzman and Brodie's values of 5% and 12%, respectively. With regard to Nor_2CPSO, direct reference values are not presently available. It is curious that

this compound—the major human nonpolar metabolite—was not discovered until 1959 (8). According to our data, the ratio of Nor_2CPSO to CPSO is about 9:1 in man and 1:5 in the dog. Based on this consideration, the nor_2sulfoxide might easily be overlooked in animal urine, but not in human urine. It seems likely that earlier studies on human urine were misinterpreted due to the use of chromatographic and spectrophotometric methods which do not differentiate CPSO from the desmethyl sulfoxides.

The 9 compounds present in Fraction *A* of human urine comprise only 7 or 8% of the drug dose. Of this sum, about 5.5% is attributable to the major metabolites in the fraction (Nor_2CPSO and Nor_1CPSO). As a result there is a balance of barely 2% for CP, CPSO and the 5 remaining metabolites. The significance of this may be appreciated by comparing the combined excretion of CP and CPSO in humans to dogs. The value estimated for man, 0.6%, is seen to be less than one-tenth the output of dogs (7.9%) (Table II). The calculation throws into sharp perspective the species dependence of chlorpromazine metabolism, and accounts in part for the apparent divergence between our earlier findings and that of other workers.

Chlorpromazine metabolism is unquestionably complex. The basis for the extensive proliferation of its metabolites lies in the biological reactivity of the chlorpromazine molecule. It appears to undergo hydroxylation with facility(9), giving rise to the compounds in Fraction *B* as well as to the glucuronic acid conjugates of the *B* components (Fraction *C*). The composition of these fractions is presumably related to the number of hydroxyl groups and their disposition in the chlorpromazine nucleus. Additional factors involved in the composition of all 3 fractions (*A*, *B* and *C*) include: susceptibility to sulfoxidation, to mono- or didemethylation, and to other as yet unidentified alterations in the nucleus and side chain. The total number of chlorpromazine metabolites was originally estimated to be 16 or more(2). Our present estimate lies closer to 24, with the final tally subject to revision as our chromatographic techniques are improved.

Summary. Man and dog are compared with reference to their metabolism of chlorpromazine following its oral administration (8 mg/kg). Both qualitative and quantitative differences are noted in the urinary metabolite patterns. Humans tend to favor the excretion of polar derivatives, along with one or 2 major nonpolar metabolites (Nor_2CPSO, 3.7% and Nor_1CPSO, 1.8%). Their output of CP (0.2%) and CPSO (0.4%) is trivial. Dogs excrete less polar material, less Nor_2CPSO (1.1%), more Nor_1CPSO (5.1%) and substantial amounts of CP (2.8%) and CPSO (5.1%). The human polar fractions contain persulfate-blue as well as persulfate-lavender staining metabolites; the blue series is conspicuously absent from dog urine.

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