(3) C. W. Liddle, *Metabolism*, **10**, 1021(1961).
(4) C. M. Kagawa, D. J. Bouska, and M. L. Anderson, *J. Pharm. Sci.*, **53**, 450(1964).
(5) C. M. Kagawa, D. J. Bouska, and M. L. Anderson, *Acta Endocrinol.*, **45**, 79(1964).
(6) C. M. Kagawa, D. J. Bouska, M. L. Anderson, and W. F. Krol, *Arch. Int. Pharmacodyn. Ther.*, **149**, 8(1964).
(7) N. Gochmann and C. L. Gantt, *J. Pharmacol. Exp. Ther.*, **135**, 312(1962).
(8) S. Ohanessian and W. W. Faloon, *Clin. Res.*, **9**, 154(1961).
(9) C. L. Gantt, N. Gochmann, and J. M. Dyniewicz, *Lancet*, **1**, 486(1961).
(10) G. Bauer, P. Rieckmann, and W. Schaumann, *Arzneim.-Forsch.*, **12**, 487(1962).
(11) S. Shaldon, J. A. Ryder, and M. Garsenstein, *Gut*, **4**, 16 (1963).
(12) G. R. Venning, in "Absorption and Distribution of Drugs," T. B. Binns, Ed., Livingstone Ltd., Edinburgh, Scotland, and London, England, 1964, pp. 150–156.
(13) G. Levy, *Lancet*, **2**, 723(1962).
(14) C. L. Gantt, N. Gochmann, and J. M. Dyniewicz, *ibid.*, **2**, 1130(1962).
(15) E. Gerhards and R. Engelhardt, *Arzneim.-Forsch.*, **13**, 972 (1963).
(16) L. Zicha, F. Weist, F. Scheiffarth, and E. Schmidt, *ibid.*, **14**, 699(1964).
(17) C. M. Kagawa, D. J. Bouska, and M. L. Anderson, *Soc. Exp. Biol. Med.*, **115**, 837(1964).
(18) H. Wagner, F. Weist, and L. Zicha, *Arzneim.-Forsch.*, **17**, 415(1967).
(19) R. T. Manning, *Clin. Res.*, **11**, 304(1963).
(20) A. Karim, R. E. Ranney, and H. I. Maibach, *J. Pharm. Sci.*, **60**, 708(1971).
(21) W. Sadée, M. Dagcioglu, and S. Riegelman, *ibid.*, **61**, 1126 (1972).
(22) W. Sadée, S. Riegelman, and S. C. Jones, *ibid.*, **61**, 1132 (1972).

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Disposition of Tritium-Labeled Spirolactones in the Dog

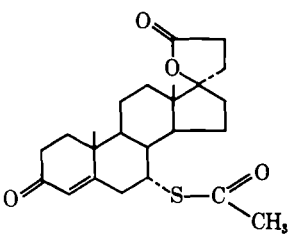
W. SADÉE*▲, S. RIEGELMAN*, and S. C. JONES†

Abstract □ The disposition and metabolism of spironolactone (I), canrenone (II), and potassium canrenoate (III) in dogs were followed by fluorescence methods and by tritium label of I and III. Spironolactone was rapidly converted to nonfluorogenic, possibly reduced, metabolites, which were concentrated in the bile fluid (35% of the dose within 6 hr.). The 4,6-dienone, III, followed a similar metabolic pathway to nonfluorogenic metabolites; however, it was eliminated at a slower rate than I. Urinary excretion was rather low (about 2% of the dose in 6 hr.), and gastric cycling was virtually absent. A preliminary pharmacokinetic model in the dog is suggested. Equimolar amounts of I and II were given perorally and intravenously for absorption studies. The bioavailability to the central compartment was about 40% following oral doses, whereas the absorption amounted to about 80% when judged by the biliary excretion after oral and intravenous doses. The difference is explained by the effective hepatic clearance following oral administration.

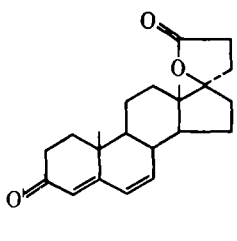
Keyphrases □ Spirolactones, fluorescent and tritium labeled—disposition, metabolism, dogs □ Spironolactone, fluorescent and tritium labeled—disposition, metabolism, dogs □ Canrenone and potassium canrenoate, fluorescent and tritium labeled—disposition, metabolism, dogs □ Absorption, spirolactones—disposition, metabolism, dogs □ Biliary excretion—spirolactones, dogs □ Radiolabeled spirolactones—disposition, metabolism, dogs □ Spectrophotofluorometry—analysis, spirolactones

The structure-activity relationships among the diuretic steroidal 17-spirolactones have been studied. The compound most potent when given orally was found to be spironolactone (I) (1–5), which is metabolized to canrenone (II) by dethioacetylation. Canrenone (II) was shown to be in rapid equilibrium with its open-chain analog, III, in dogs (6). *In vitro* studies with the small gut mucosa of rats revealed an effective hydrolytic cleavage of the γ-lactone ring of II at this site (7).

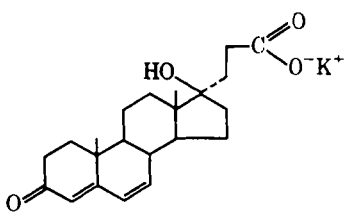
Earlier workers (4) suggested that either I or a metabolite of I other than II may also contribute to the pharmacological effect. However, no significant difference in activity between I and the potassium salt of canrenoic acid, III, was found following oral and



I



II



III

parenteral administration (8, 9), although III exists in rapid equilibrium with II *in vivo* (6).

A rapid reduction of the 4-en-3-one function has been proved for a variety of steroids and would presumably cause a loss of activity of the spirolactones (10). The high oral activity of spironolactone was, therefore, explained by inhibition of the metabolic reduction by the presence of the 7α-thioacetyl group (7). No data have been reported on the metabolic fate of the 4,6-dien-3-one moiety present in II. Only about 1% of an oral dose of 1 g. of I was excreted in the urine as II in man (7) when measured by the fluorescence assay of Gochmann and Gantt (11).

Specific fluorometric assays have been reported which enable differential assessment of I, II, and III (12). Since reduced metabolites would not fluoresce, $C_{20,21}$-tritium-labeled I and III were studied to estimate unknown metabolites.

To account for the high oral activity of I, II, and III compared to other spirolactones, equivalent doses of I, II, and III were administered by the oral route and intravenously to female dogs.

EXPERIMENTAL

Fluorescence Assays—See *Reference 12.*

Preparation of Dogs—See *Reference 6.*

Radioactive Materials—Compound I, tritium labeled at $C_{20,21}$ with a specific activity of 0.59 mc./mg., was used[1].

Compound ^{3}H-III was prepared by heating I with 0.1 *N* KOH 10 min. at 90°. This procedure could be shown to convert quantitatively I to III without causing an exchange of the tritium label at $C_{20,21}$. Both ^{3}H-I and ^{3}H-III were found to be radiochemically pure using TLC.

A 100-mg. dose of ^{3}H-I and the equimolar amount of ^{3}H-III (95 mg.) containing approximately 0.4–1.0 mc. of radioactive material was administered intravenously and orally.

Reagents—Internal standard: ^{3}H-toluene (2.26×10^6 d.p.m./ml.). (*a*) Toluene cocktail for nonaqueous samples[2]. (*b*) Dioxane cocktail for aqueous samples, according to Bray (13). (*c*) Solubilizer for plasma samples[3].

Radioactive Assay Procedures—*Plasma*—To 0.2 ml. plasma, 0.5 ml. of the solubilizer was added, and the mixtures were heated for 45 min. at 60°. After cooling, 10 ml. of the dioxane cocktail was added.

Bile—To 0.01 ml. bile, 10 ml. of dioxane cocktail was added.

Urine—To 0.01 ml. urine, 10 ml. of the dioxane cocktail was added.

The samples were placed in plastic vials and counted by a liquid scintillation counter[4] for 10 min. (bile samples for 1 min.). A second reading was performed after adding the ^{3}H-toluene standard.

RESULTS AND DISCUSSION

In a previous study (6), the disposition kinetics of I, II, and III in dog plasma were described using fluorometric procedures (12). To assess nonfluorogenic metabolites, tritium-labeled I and III were administered intravenously and I was administered perorally.

After intravenous administration of 100-mg. doses of ^{3}H-I to dogs, the data shown in Fig. 1 were obtained by radioactive and fluorescent analyses of the blood plasma samples. The differences between the fluorogenic metabolites (12) (total fluorescence assay) and radioactivity resulted in a curve representing the concentration of nonfluorogenic metabolites. It is apparent that I undergoes rapid metabolic conversion, possibly to reduced metabolites. The increase in concentrations of total radioactivity and total fluorescence over the 1st hr. after injection indicates the formation of metabolites with a smaller volume of distribution than I.

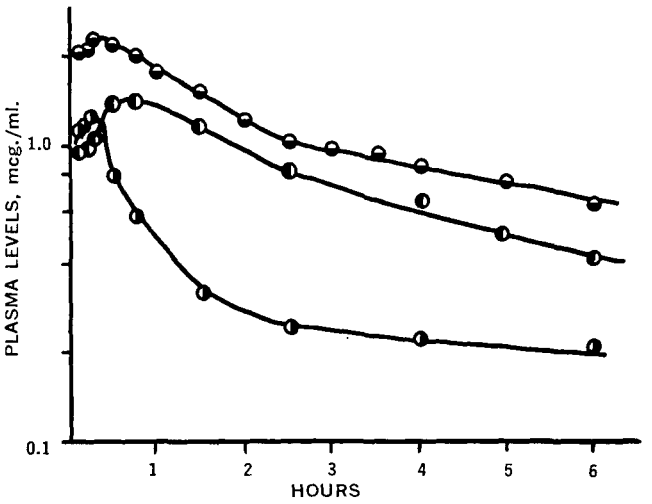


Figure 1—*Plasma levels after intravenous administration of 100 mg. ^{3}H-I to Dog 15871. Key: ◒, total radioactivity; ◐, total fluorescent concentration; and ◑ (◒ − ◐), nonfluorogenic metabolites. All concentrations are expressed as micrograms of II per milliliter.*

The cumulative biliary excretion is shown in Fig. 2 following 100 mg. ^{3}H-I i.v. The total radioactivity accounted for 35.5% of the dose during a 6-hr. collection period, in contrast to only 3% fluorogenic metabolites ("total fluorescence assay") (12). Upon extrapolating the excretion curves to an infinite time, one could estimate a total biliary excretion of about 60% of the administered dose. The nonfluorogenic metabolites were not extractable with ether, indicating conjugation to polar metabolites, which were then concentrated in the bile.

In one experiment, the bile cannula was removed after the initial period of 6 hr. to allow hepatic cycling, and blood was sampled for 4 days. Plasma levels of total radioactivity were as high as 0.19

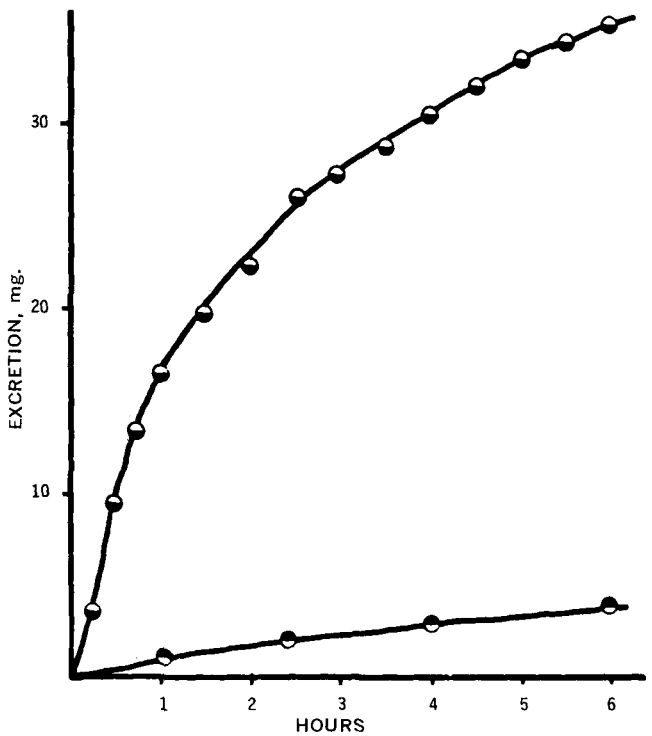


Figure 2—*Cumulative biliary excretion following an intravenous dose of 100 mg. of ^{3}H-I to Dog 15871. Key: ◒, total radioactivity; and ◓, total fluorescence. All amounts are expressed as milligrams of I.*

[1] G. D. Searle and Co., Chicago, Ill.
[2] 5 g. Omnifluor/1 l. toluene liquid scintillation grade, Eastman Chemical Co.
[3] 0.75 *M* Soluene, Packard Co.
[4] Packard LSS 3380 AAA.

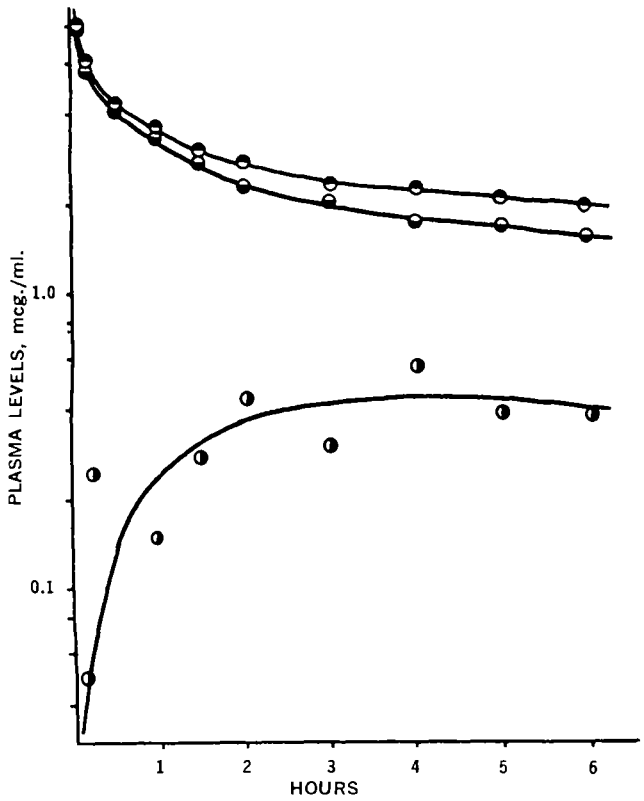


Figure 3—*Plasma levels after an intravenous dose of 95 mg. ^{3}H-III to Dog 16526. Key: ◓, total radioactivity; ◒, total fluorescent concentration; and ◑ (◓ − ◒), nonfluorogenic metabolites. All concentrations are expressed as micrograms of II per milliliter.*

mcg./ml. after 80 hr. One can estimate a disposition half-life of 20 hr. for this time interval.

Since the metabolic fate of the dienone moiety, represented by II and III, has not been reported, tritium-labeled III was investigated by the same procedure as for I. The results are shown in Fig. 3. A

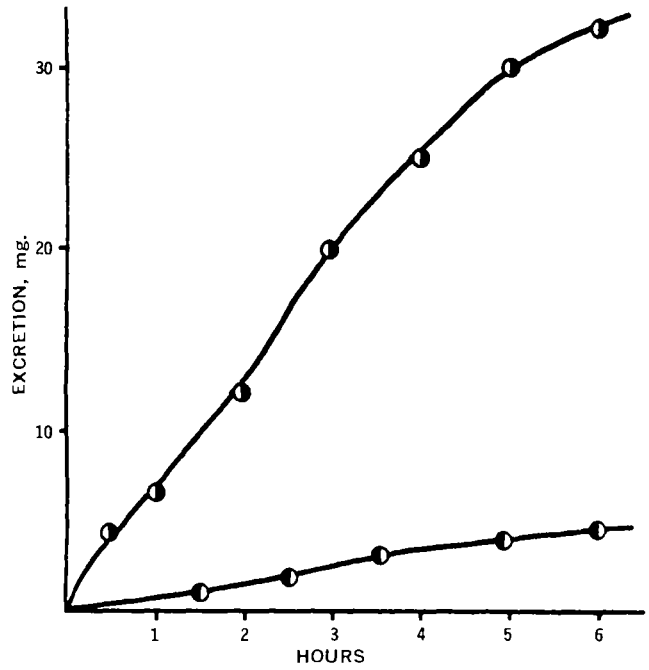


Figure 4—*Cumulative biliary excretion following an intravenous dose of 95 mg. ^{3}H-III to Dog 16526. Key: ◑, total radioactivity; and ◐, total fluorescence. All amounts are expressed as milligrams of I.*

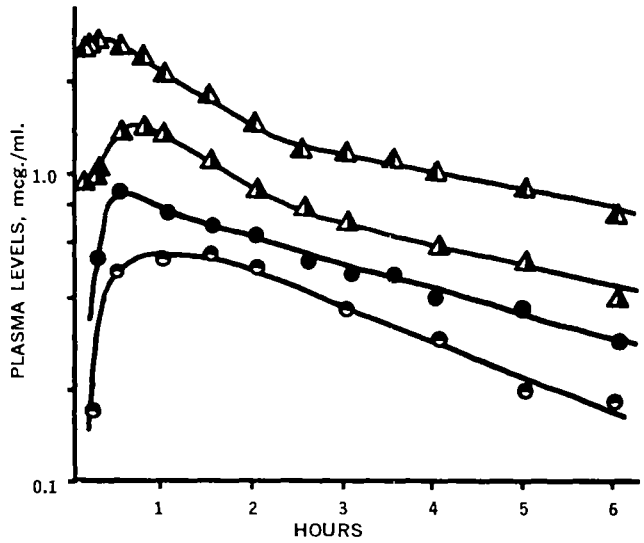


Figure 5—*Plasma levels after an intravenous dose (triangles) and an oral dose (circles) of a polyethylene glycol 400 solution of 100 mg. ^{3}H-I to Dog 15871. Key: ▲ and ●, total radioactivity; and △ and ◓, total fluorescent concentration. All concentrations are expressed as micrograms of II per milliliter.*

small concentration of nonfluorogenic metabolites in plasma has to be postulated over a period of 6 hr. postadministration. However, the fluorogenic metabolites accounted for only 2–4% in the bile, whereas the total radioactivity accounted for about 32% of the dose within 6 hr. (Fig. 4). Again, nonfluorogenic, conjugated metabolites were concentrated in the bile fluids. Examination of Figs. 2 and 4 indicate that III is converted to nonfluorogenic metabolites and excreted into the bile at an overall rate lower than is found for I when comparing these two dogs.

The ester glucuronide of III was reported to be a major metabolite in man (14). In the dog, less than 1% of the dose could be accounted

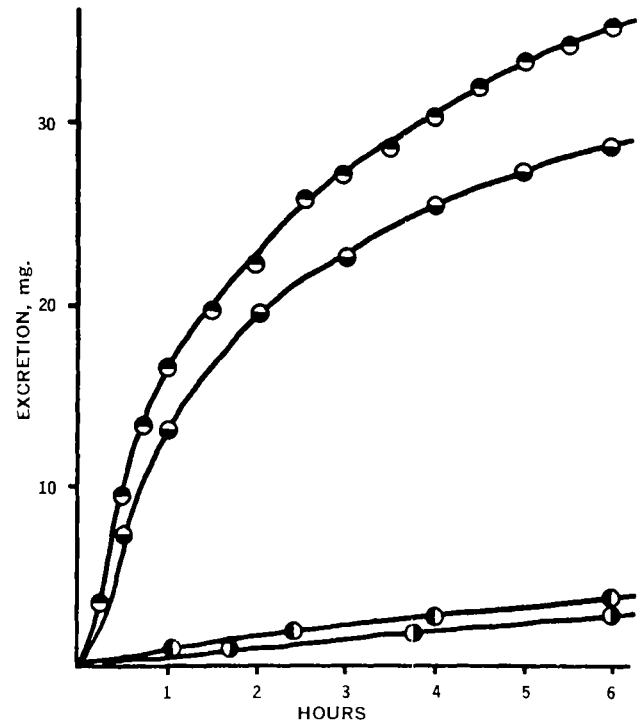


Figure 6—*Cumulative biliary excretion after an intravenous dose and an oral dose of ^{3}H-I to Dog 15871. Key: ◓ and ◒, total radioactivity after intravenous and oral administration, respectively; and ◐ and ◑, total fluorescence after intravenous and oral administration, respectively. All amounts are expressed as milligrams of I.*

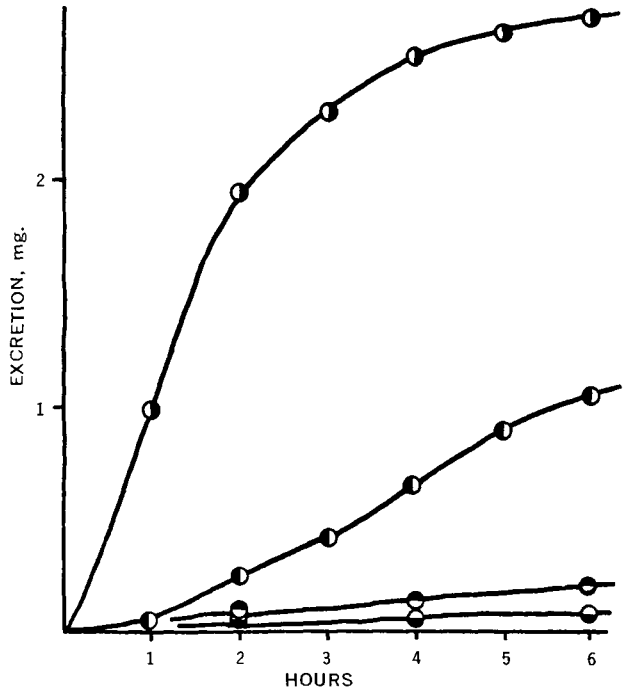


Figure 7—*Cumulative urinary excretion following an intravenous dose and an oral dose of 100 mg. ^{3}H-I to Dog 15871. Key: ◑ (intravenous) and ◐ (oral), total radioactivity; and ◓ (intravenous) and ◒ (oral), total fluorescence. All amounts are expressed as milligrams of I.*

for as the glucuronide by fluorescence analysis (12), which was excreted into the biliary system. No polar conjugates were detectable in plasma by fluorescence assay. Thus, the metabolic fate of the spirolactones is clearly different in the dog when compared with man.

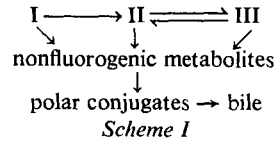
Absorption Studies—Figure 5 shows the plasma curves following oral and intravenous doses of radioactive I to the same dog assayed by the appropriate fluorescence (12) and ^{3}H-radioactivity procedures. Comparison of the radioactivity curve indicated an oral availability of I to the central compartment of about 40% of the dose. The difference between the fluorescence assay and the total radioactivity following the oral dose again indicated the presence of nonfluorogenic metabolites, as discussed for the intravenous administration of I (Fig. 1).

Different definitions of oral availability can be invoked, such as comparison of the bile, blood, and urine levels (15). On oral administration, all of the drug is usually absorbed *via* the hepatic portal system. If metabolism or biliary secretion precede the transit into the central compartment, a lower estimate of drug availability will be seen by comparison of plasma levels from oral and intravenous administrations. The cumulative biliary excretion curves after administration of I by the oral and intravenous routes are compared in Fig. 6. These data indicate the degree of absorption under the condition of the study to be approximately 80%.

Unlike the biliary excretion, the renal excretion reflects the availability of I in the plasma. Thus, the amount of radioactivity excreted following oral administration accounts for about 40% of an equivalent intravenous dose. The ratios between fluorogenic and nonfluorogenic metabolites in urine (Fig. 7) were similar in value to what is found in bile. An effective renal tubular reabsorption of II and III is probable.

The total urinary excretion is remarkably low (about 1% of a peroral dose of 100 mg. I within 6 hr.). Although hepatic cycling was made possible by removing the bile cannula after 6 hr., the urinary excretion did not exceed 2% over a collection period of 80 hr. In contrast, urinary excretion in man accounted for about 47% of the dose within 5 days (14). The metabolism of the tritiated side chain on C_{17} must be considered; however, it is likely that the majority of the drug is excreted *via* the feces as conjugates.

Gastric Secretion—Gastric fluids were collected through a gastric fistula. The output of gastric fluids varied widely from dog to dog. Some impact of I and II on gastric flow was found; however, it was



Scheme I

not statistically significant. The secretion of sodium, potassium, and hydrogen ions was not stimulated. Following intravenous doses of ^{3}H-I and ^{3}H-III, only low amounts could be detected (about 10 mcg. or about 0.01% of the dose over 6 hr.), although maximal flow rates of gastric fluids up to 150 ml./hr. were produced. A virtual absence of gastric cycling can be postulated.

CONCLUSIONS

The model shown in Scheme I can be suggested so far for the metabolism and the excretion of I. Preliminary studies showed that the predominant part of metabolites in the bile were polar conjugates of nonfluorogenic metabolites. More work concerning the nature of metabolites will be reported soon.

Spironolactone (I) itself is unlikely to contribute to the diuretic activity, since it is virtually cleared from plasma within 1 hr. (6). Since the 4-en-3-one derivatives of the 17-spirolactone series are not deprived of antimineralocorticoid activity when given parenterally, it can be assumed that the activity is not necessarily correlated to the dienone moiety of II and III. On the other hand, the enone seems to be cleared to a large extent from the plasma by metabolic reduction in the liver and by biliary excretion, which could explain the low oral activity of many derivatives.

The high oral activity of I may be explained in part by dethioacetylation to the dienone II, which is metabolized with a slower rate than I and escapes an effective hepatic clearance. However, metabolites of I with a diuretic activity higher than that of II and III could account for the pharmacological effect of I in dogs.

REFERENCES

(1) C. L. Gantt, *N. Y. J. Med.*, **61,** 756(1961).

(2) C. M. Kagawa, *Endocrinology*, **67,** 125(1960).

(3) J. A. Cella and R. C. Tweit, *J. Org. Chem.*, **24,** 1109(1959).

(4) C. M. Kagawa, D. J. Bouska, and M. L. Anderson, *Soc. Exp. Biol. Med.*, **115,** 837(1964).

(5) R. C. Tweit, F. B. Colton, N. L. McNiven, and W. Klyne, *J. Org. Chem.*, **27,** 3325(1962).

(6) W. Sadée, S. Riegelman, and S. C. Jones, *J. Pharm. Sci.*, **61,** 1129(1972).

(7) E. Gerhards and R. Engelhardt, *Arzneim.-Forsch.*, **13,** 972(1963).

(8) R. T. Manning, *Clin. Res.*, **11,** 304(1963).

(9) C. M. Kagawa, D. J. Bouska, M. L. Anderson, and W. F. Krol, *Arch. Int. Pharmacodyn. Ther.*, **149,** 8(1964).

(10) J. Cella, E. A. Brown, and R. R. Burtner, *J. Org. Chem.*, **24,** 743(1959).

(11) N. Gochmann and C. L. Gantt, *J. Pharmacol. Exp. Ther.*, **135,** 312(1962).

(12) W. Sadée, M. Dagcioglu, and S. Riegelman, *J. Pharm. Sci.*, **61,** 1126(1972).

(13) G. A. Bray, *Anal. Biochem.*, **1,** 279(1960).

(14) A. Karim, R. E. Ranney, and H. I. Maibach, *J. Pharm. Sci.*, **60,** 708(1971).

(15) P. A. Harris and S. Riegelman, *ibid.*, **58,** 71(1969).

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