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Spironolactone- and Canrenone-Induced Changes in Hepatic (Na^+,K^+)ATPase Activity, Surface Membrane Cholesterol and Phospholipid, and Fluorescence Polarization in the Rat

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We studied changes in hepatic membrane (Na^+,K^+)ATPase activity and membrane lipids induced by canrenoate, the water-soluble congener of canrenone, the active metabolite of spironolactone. (Na^+,K^+)ATPase activity was decreased after canrenoate in a dose- and time-dependent manner. Decreased activity was demonstrated at the lowest dose (91% of control after 5 μmoles per 100 gm body weight per day × 3 days); the maximum dose (30 μmoles per 100 gm body weight per day × 3 days) resulted in activity 38% of untreated control values. A 20 μmoles per 100 gm body weight per day dose decreased enzyme activity to 89 and 55% of control after 24 and 72 hr, respectively. The nonionic detergent Triton WR-1339 partially reversed drug-induced inhibition, suggesting that the enzyme changes may be related to altered membrane lipids.

Membrane cholesterol increased 17% after 3 days of 30 μmoles canrenoate per 100 gm body weight per day; phospholipids decreased by 12%. The cholesterol to phospholipid molar ratio increased from 0.419 to 0.555. Membrane fluidity, as measured by the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene decreased after treatment with 20 μmoles canrenoate per 100 gm body weight per day for 3 days.

These results describe *in vivo* and *in vitro* inhibition of hepatic (Na^+,K^+)ATPase activity. Increased membrane cholesterol with decreased phospholipid alters membrane fluidity and may be partially responsible for the change in (Na^+,K^+)ATPase activity.

Spironolactone-related changes in biliary physiology and hepatocyte metabolism are clinically relevant since patients with cirrhosis complicated by ascites are often effectively treated with this drug. Spironolactone is rapidly converted to its active metabolite, canrenone, by dethioacetylation (1). Some hepatic effects of spironolactone in the rat include (i) increase in bile flow due to increased excretion of biliary water independent of bile acids (2, 3); (ii) increased transport maximum of bromosulfophthalein (4); (iii) doubling of biliary cholesterol excretion (3); (iv) increased activity of bilirubin-UDP-glucuronyl transferase, ethylmorphine-*N*-demethylase, aniline hydroxylase, and cytochrome-C reductase (5), and (v) accelerated biotransformation or elimination of numerous drugs (5). In addition to metabolic alterations in the experimental animal, spironolactone induces the activity of several hepatic enzymes in patients with alcoholic cirrhosis (6).

Hepatic sodium-potassium adenosine triphosphatase [(Na^+,K^+)ATPase] is an important transport enzyme which is sensitive to changes in the lipid content of the membrane. Functionally, it is important in osmoregulation of cell volume, maintaining an intracellular environment favorable for protein synthesis, and generating an electrochemical gradient to provide the energy necessary

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for hepatic uptake through sodium symport systems (7–10). In heart muscle preparations, canrenone inhibits (Na^+,K^+)ATPase by competitive binding at the ouabain binding site (11). Because of the importance of (Na^+,K^+)ATPase in hepatocyte physiology, we sought to explore the *in vivo* effect of spironolactone and its active metabolite on (Na^+,K^+)ATPase activity in isolated liver surface membranes (LSMs), liver surface cholesterol and phospholipid content, and membrane fluidity as measured by fluorescence polarization. We also studied the *in vitro* effect of spironolactone, canrenone, and potassium-canrenoate on (Na^+,K^+)ATPase activity, and possible mechanisms for the changes in (Na^+,K^+)ATPase activity.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 160 to 225 gm (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were used. Animals were housed in the animal care facility with cellulose corn cob bedding (Bed-o-Cob, Anderson Co., Maumee, Ohio) and alternating 12-hr light and dark cycles. Free access to laboratory chow (Ralston Purina Co., St. Louis, Mo.) and water was allowed. Double-glass distilled water was used for all reagents. Adenosine-5-triphosphate, adenosine-5-monophosphate, glucose-6-phosphate, ouabain, and Triton WR-1339 were obtained from Sigma Chemical Co., St. Louis.

LSM fractions were prepared by the method of Neville (12). The purity and distribution of hepatic marker enzymes in this membrane fraction were determined by relative enrichment of 5′-nucleotidase, magnesium ATPase (Mg^{++}-ATPase), and glucose-6-phosphatase.

Enzyme Assays

(Na^+,K^+)ATPase (ATP-phosphohydrolase, E.C. 3.6.1.3) activity was determined in liver homogenates and LSM fractions after freeze thawing by the method of Ismail-Beigi and Edelman (13) with slight modification for LSM fractions. The incubation medium was warmed to 37°C for 5 min prior to initiation of the enzyme reaction by addition of 0.1 ml of sample (final protein concentration 200 to 250 μg per ml). Total ATPase activity was determined at pH 7.4 in a final volume of 2 ml containing ATP (5.0 m*M*), Mg^{++} (5.0 m*M*), Na^+ (120 m*M*), K^+ (12.5 m*M*), Tris (125 m*M*), Cl^- (137.5 m*M*), and EGTA (1.0 m*M*). (Na^+,K^+)ATPase activity was determined by the difference between total ATPase and activity remaining after addition of ouabain (10^{-3} m*M*). The reaction was terminated by addition of 0.5 ml of ice-cold 30% trichloroacetic acid to the reaction mixture. For experiments, *in vitro* spironolactone and canrenone were dissolved in ethanol and water. The final concentration of ethanol in the reaction medium was maintained at 8.14 m*M* in all assay tubes including blanks and controls. Canrenoate was dissolved in water only and compared with separate controls. Membrane purity was assessed by 5′-nucleotidase activity (E.C. 3.1.3.5) (14), and Mg^{++}-ATPase activity (E.C. 3.6.3.5) (15). Microsomal contamination was monitored by glucose-6-phosphatase activity (E.C. 3.1.3.9) (16).

The amount of inorganic phosphate released in each assay was determined by the Fiske-Subbarow assay (17). All homogenate samples were stored in 1 m*M* sodium bicarbonate at −20°C until assayed. LSM fractions were stored in 1 m*M* sodium bicarbonate for (Na^+,K^+)ATPase and in 0.01 *M* Tris buffer (pH 7.4) for the other enzyme assays. (Na^+,K^+)ATPase was measured within 24 hr, other enzymes within 48 hr. Proteins were determined by the method of Lowry et al. (18) using crystalline bovine serum albumin as a standard.

Membrane Cholesterol and Phospholipids

Lipids were extracted from an aliquot of surface membrane containing 1 mg of protein using the chloroform-methanol extraction described by Folch et al. (19). Prior to lipid extraction, an internal standard, coprostanol (100 μg per mg protein), was added to all samples used for cholesterol determination. Half of each extraction was used for phospholipid determination (20). The remainder of the extracted lipid containing the internal standard was used to determine cholesterol by gas/liquid chromatography using a Model 3920 B Perkin Elmer GL chromatograph (21).

Fluorescence Polarization

Fluorescence polarization was measured using the fluorescent hydrocarbon 1,6-diphenyl-1,3,5-hexatriene (DPH). Membrane protein was added to 2 ml 10 m*M* Hepes and 50 m*M* NaCl (pH 7.4) to obtain to a final protein concentration of 37.5 μg per ml. DPH (1 m*M* in tetrahydrofuran) was added to the vigorously vortexed sample by a Hamilton microliter syringe to achieve a phospholipid to DPH mole ratio of approximately 1,000. Fluorescence polarization was measured in a SLM Instrument Model 8000 spectrofluorometer with excitation at 357 nm and detection at 435 nm in the presence of a Corning CS 3-73 cutoff filter. Measurements were made at 25°C. Polarization was calculated from the fluorescence intensities parallel ($I_\parallel$) and perpendicular ($I_\perp$) to the direction of polarization of the exciting radiation $[P = [(I_\parallel/I_\perp) - 1]/[(I_\parallel/I_\perp) + 1]]$ (22). Spectral scans of membranes from untreated and treated animals were done to permit comparison of fluorescent intensities.

Drugs

Spironolactone (SC 9420), canrenone (SC 9376), and K^+ canrenoate (SC 14266) were gifts from G. D. Searle Co. Spironolactone was dissolved in corn oil and administered by gavage in doses of 10, 20, or 30 μmoles per 100 gm body weight per 24 hr in volumes no greater than 1 cm^3 per dose. Controls were given 1 cm^3 of corn oil by gavage. Canrenoate K^+ was dissolved in sterile water and given intraperitoneally in doses of 5, 10, 20, or 30 μmoles per 100 gm body weight per 24 hr. Total volume injected was 1 cm^3. The last dose was given 24 hr prior to beginning membrane preparation.

Statistics

Statistical differences between different groups were determined by Student's t test. In experiments evaluating dose response, time dependence, effect of Triton WR-

1339, and membrane lipids, one way analysis of variance was used to determine significance; Dunnett's multiple range procedure was used to determine the values different than control (23).

RESULTS

Effect of Increasing Canrenoate Doses on Hepatic Surface Membrane (Na^+,K^+)ATPase Activity (Figure 1)

Canrenoate was given intraperitoneally in daily doses of 5.0, 10.0, 20.0, and 30.0 μmoles per 100 gm body weight 3 days prior to decapitating the animals. All doses reduced (Na^+,K^+)ATPase activity ($p < 0.01$) when compared with values in control animals by Dunnett's multicomparison procedure. The largest dose decreased enzyme activity to 38% of control. Equimolar daily doses of the parent compound, spironolactone, decreased (Na^+,K^+)ATPase activity nearly identical to changes after canrenoate; enzyme activity was 35% of control after 30 μmoles spironolactone per 100 gm body weight per day for 3 days (data not shown). Surface membrane enzyme enrichment in untreated animals and after maximum canrenoate and spironolactone doses (Table 1) revealed equal enrichment of (Na^+,K^+)ATPase, Mg^{++}-ATPase, and 5′-nucleotidase and minimal microsomal contamination, as indicated by the glucose-6-phosphatase values.

Time Dependence of Canrenoate Treatment on Hepatic Surface Membrane (Na^+,K^+)ATPase Activity (Figure 2)

Hepatic (Na^+,K^+)ATPase activity progressively decreased 24, 48, and 72 hr after canrenoate doses of 20 μmoles per 100 gm body weight per day. Each value was significantly lower than the control value ($p < 0.01$) by Dunnett's multicomparison procedure with maximum inhibition after 72 hr of treatment with enzyme activity 55% of control. A similar experiment with fewer animals ($n = 3$) treated with spironolactone (20 μmoles per 100 gm body weight per day) resulted in (Na^+,K^+)ATPase activity 53% of control after 72 hr.

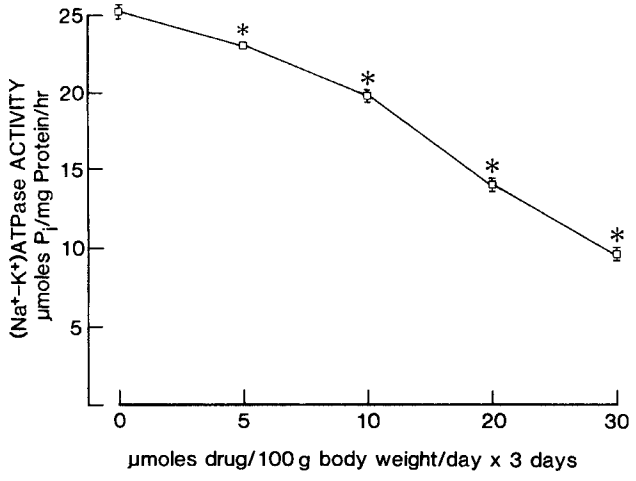

FIG. 1. Effect of increasing doses of canrenoate on hepatic (Na^+,K^+)ATPase activity. Doses indicated were given by intraperitoneal injection each day for 3 days. Values represent the mean ± 1 S.E. of six separate animals. All points are significantly different than control ($p < 0.01$) by Dunnett's multicomparison test.

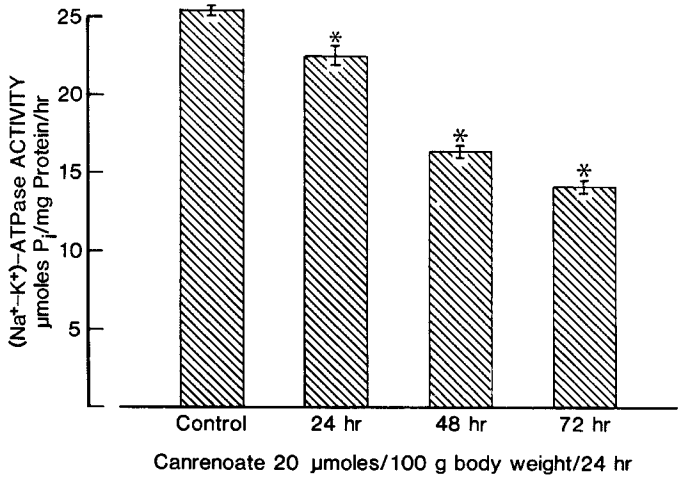

FIG. 2. Time-dependent effect of 20 μmoles canrenoate per 100 gm body weight per day. Values represent the mean ± 1 S.E. of six separate animals. All points are significantly different than control ($p < 0.01$) by Dunnett's multicomparison test.

TABLE 1. Effect of Canrenoate on (Na^+,K^+)ATPase, Mg^{++}-ATPase, 5′-Nucleotidase, and Glucose-6-Phosphatase[a]

	(Na^+K^+)ATPase	Mg^{++}-ATPase	5′-Nucleotidase	Glucose-6-phosphatase
Control				
Liver homogenate	2.1 ± 0.2	3.7 ± 0.1	4.4 ± 0.2	7.3 ± 0.2
LSM	24.9 ± 0.5	49.5 ± 1.7	64.5 ± 1.3	1.6 ± 0.2
Enrichment ratio	11.8 ± 1.1	13.3 ± 0.5	14.8 ± 0.5	0.22 ± 0.04
(LSM/LH)	(5)	(5)	(3)	(3)
Canrenoate				
Liver homogenate	0.8 ± 0.1	3.6 ± 0.1	4.9 ± 0.4	6.9 ± 0.2
LSM	9.6 ± 0.3	48.5 ± 0.9	63.3 ± 1.3	1.6 ± 0.3
Enrichment ratio	12.3 ± 0.9	13.5 ± 0.2	13.1 ± 1.2	0.23 ± 0.04
(LSM/LH)	(5)	(5)	(5)	(5)

[a] Canrenoate dose was 30 μmoles/100 gm body weight/day × 3 days. Activity is in μmoles Pi/milligram protein/hour. Values represent the mean ± 1 S.E. with the number of experimental animals indicated by the number in parentheses. Enrichment ratios represent the mean ± 1 S.E. of the enzyme activities of eash LSM divided by the enzyme activity of the corresponding liver homogenate (LH).

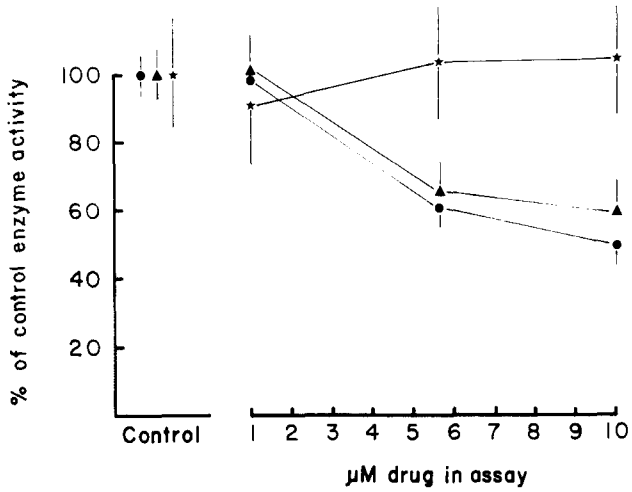

FIG. 3. *In vitro* effect of canrenoate (★), canrenone (▲), spironolactone (●) on (Na^+,K^+)ATPase activity. Values represent the mean ± 1 S.E. for 6 canrenoate, 3 canrenone, and 3 spironolactone enzyme inhibition curves.

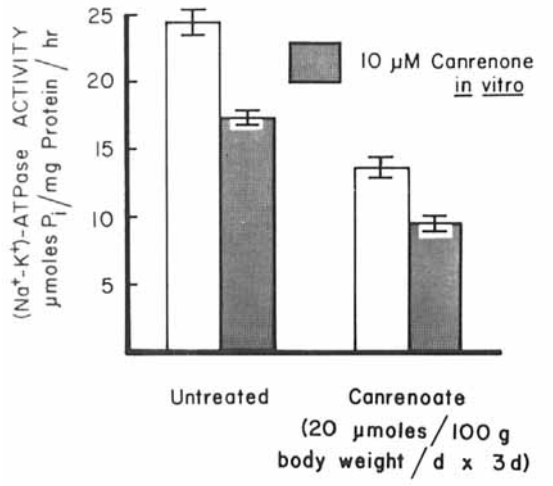

FIG. 4. *In vitro* effect of canrenone on (Na^+,K^+)ATPase activity in membranes from untreated and canrenoate and six treated animals. The differences between the control valves (*open bars*) and the *in vitro* canrenone values (*shaded bars*) are significant by a Student's t test, $p < 0.0001$.

EFFECT OF CANRENOATE, CANRENONE, AND SPIRONOLACTONE ON LSM (Na^+,K^+)ATPASE *IN VITRO* (FIGURE 3)

The effects of canrenoate, its lipid-soluble form, canrenone, and their parent compound, spironolactone, on (Na^+,K^+)ATPase were evaluated in a series of experiments *in vitro*. Incubation of hepatic surface membrane with increasing concentrations of the lipid-soluble congeners (canrenone and spironolactone) resulted in a concentration-dependent fall in enzyme activity. Canrenoate, the water-soluble hydroxycarboxylic acid form of canrenone, did not significantly effect (Na^+,K^+)ATPase activity *in vitro* (Figure 3). Since the effect of these compounds on (Na^+,K^+)ATPase could be due solely to physical interaction with the enzyme, this experiment *in vitro* was repeated in membranes from animals treated for 3 days with 20 μM canrenoate per 100 gm body weight per day (Figure 4). Similar decreases in enzyme activity were noted with 10 μM canrenone in membranes isolated from untreated rats [25.3 ± 0.7 to 16.0 ± 0.4 (63%)] and rats treated with 20 μmoles canrenoate per 100 gm body weight per day for 3 days [14.0 ± 0.4 to 9.6 ± 0.2 (69%)]. To rule out possible interference with the Fiske-Subbarow assay, the assay was repeated with a known concentration of inorganic phosphorus in the presence and absence of spironolactone; no differences were detected.

EFFECT OF TRITON WR-1339 ON (Na^+,K^+)ATPASE ACTIVITY IN HEPATIC SURFACE MEMBRANE FRACTIONS FROM UNTREATED AND CANRENOATE-TREATED ANIMALS (FIGURE 5)

The nonionic detergent Triton WR-1339 was added in increasing concentration to the (Na^+,K^+)ATPase enzyme assay media prior to analysis of enzyme activity in membranes isolated from untreated animals and from canrenoate-treated animals (20 μmoles per 100 gm body

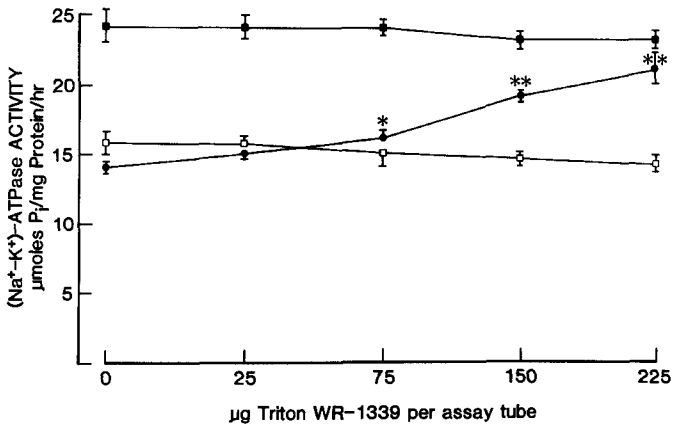

FIG. 5. Effect of Triton WR-1339 on (Na^+,K^+)ATPase activity. Values represent the mean ± 1 S.E. Membranes from untreated rats (■, n = 5) and untreated rats with 10 μM *in vitro* canrenone (□, n = 7) are unchanged by Triton WR-1339. Activity in membranes from rats treated with 20 μmoles canrenoate per 100 gm body weight per day × 3 days (●, n = 6) is significantly greater than control (*, $p < 0.05$; **, $p > 0.01$) using Dunnett's multicomparison test.

TABLE 2. EFFECT OF INCREASING DOSES OF CANRENOATE ON HEPATIC SURFACE MEMBRANE CHOLESTEROL, PHOSPHOLIPID, AND MOLAR CHOLESTEROL PHOSPHOLIPID RATIO[a]

Canrenoate dose	Cholesterol	Phospholipid	Cholesterol: phospholipid molar ratio
	(mmoles membrane lipid per μg protein)		
Untreated	0.226 ± 0.012	0.541 ± 0.016	0.419 ± 0.025
5 μmoles	0.236 ± 0.012	0.533 ± 0.012	0.443 ± 0.023
10 μmoles	0.240 ± 0.018	0.513 ± 0.017**	0.470 ± 0.039?*
20 μmoles	0.247 ± 0.016**	0.498 ± 0.015**	0.498 ± 0.041**
30 μmoles	0.265 ± 0.017**	0.478 ± 0.010**	0.555 ± 0.029**

[a] Values indicate mean ± 1 S.D. with six animals in each group. Canrenoate dose indicates the number of micromoles canrenoate given per 100 gm body weight each day for 3 days. Significant differences between groups were determined by analysis of variance followed by Dunnett's multicomparison procedure: *, $p < 0.05$; **, $p < 0.01$.

TABLE 3. EFFECT OF CANRENOATE ON HEPATIC SURFACE MEMBRANE LIPIDS AND FLUORESCENCE POLARIZATION[a]

	Cholesterol	Phospholipid	Cholesterol: phospholipid molar ratio	Fluorescence polarization
	(mmoles membrane lipid per μg protein)			
Untreated	0.235 ± 0.011	0.534 ± 0.015	0.440 ± 0.019	0.252 ± 0.009
Canrenoate 20 μmoles/100 gm body weight/day × 3 days	0.276 ± 0.018	0.484 ± 0.013	0.569 ± 0.029	0.264 ± 0.007
Significance	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.01$

[a] Values indicate the mean ± 1 S.D. with six animals in each experimental group. Statistical differences between groups were determined by Student's t test.

weight per day for 3 days). In addition, a series of assays was done using membranes from untreated rats in the presence of canrenone (10 μM) and increasing concentrations of Triton WR-1339. Triton WR-1339 had no effect on enzyme activity in membranes from untreated animals in the presence or absence of canrenone *in vitro*. In canrenoate-treated animals, addition of Triton WR-1339 in amounts of 75 μg and greater increased enzyme activity. In the presence of 255 μg Triton WR-1339, (Na^+,K^+)ATPase activity increased from 14.0 ± 0.4 to 21.4 ± 1.0 μmoles Pi per mg protein per hour. This value represents 153% of activity before addition of Triton WR-1339 and 91% of enzyme activity from the surface membrane of the corresponding untreated animal.

EFFECT OF CANRENOATE DOSE ON MEMBRANE LIPIDS

Increasing daily doses of canrenoate progressively increased LSM total cholesterol content (Table 2). Using analysis of variance followed by the Dunnett's multicomparison procedure, membrane cholesterol values after canrenoate doses of 20 and 30 μmoles per 100 gm body weight per day for 3 days were statistically greater ($p < 0.01$) than untreated controls. Membrane phospholipid values decreased progressively. Canrenoate doses increased reaching $p < 0.01$ significance at 10 μmoles per 100 gm body weight canrenoate dose. Cholesterol to phospholipid ratios were determined individually for each membrane preparation. Increasing membrane cholesterol to phospholipid ratios correlated with increasing canrenoate doses achieving statistical significance ($p < 0.05$) after the canrenoate dose of 10 μmoles per 100 gm body weight per day for 3 days.

CANRENOATE EFFECT ON MEMBRANE FLUIDITY

Membrane fluidity was measured by fluorescence polarization using DPH as a membrane probe in untreated controls and in animals treated with 20 μmoles canrenoate per 100 gm body weight per day for 3 days. Repeat membrane lipids (Table 3) confirmed the findings in the dose response curve although the membrane cholesterol value in this set of experimental values was higher than the corresponding value in the dose response curve. Emission and excitation fluorescence scans were done on control and treated membranes to obtain assurance that intrinsic protein fluorescence or membrane bound drug did not interfere with DPH fluorescence measurements and that fluorescent intensities would permit comparison. A significant ($p < 0.01$) increase in fluorescence polarization after canrenoate treatment is consistent with changes in membrane fluidity predicted by membrane lipid alterations.

DISCUSSION

The effects of spironolactone on the liver are known to include: increased bromosulfophthalein transport maximum, bile salt-independent bile flow, and biliary cholesterol secretion, smooth endoplasmic reticulum proliferation, induction of many enzymes, and accelerated drug metabolism (2, 3, 5, 24). We investigated the effect of spironolactone and canrenone on hepatic (Na^+,K^+)ATPase because of the important role of the sodium pump on metabolism and transport.

In rats treated with canrenoate and spironolactone, there is a dose- and time-dependent decrease in hepatic (Na^+,K^+)ATPase activity. Using Dunnett's multicomparison statistic, the lowest dose tested (5 μmoles per 100 gm body weight per day × 3 days) and the shortest time interval (24 hr after one 20 μmole per 100 gm body weight dose) significantly decreased hepatic (Na^+,K^+)ATPase activity. Many cellular effects of steroid hormones are mediated through new protein synthesis modulated by specific cytosol receptors. It is unlikely that the fall in hepatic (Na^+,K^+)ATPase activity is mediated by competition of canrenoate or spironolactone for cytoplasmic mineralocorticoid receptors since these receptors are apparently not present in liver (25). Possible mechanisms for decreased hepatic (Na^+,K^+)ATPase activity induced by these steroid derivatives include: alterations in serum or total body potassium; decreased membrane fluidity due to membrane lipid changes; decreased serum corticosterone; incorporation of the drug in the membrane; drug overlap with other steroid receptors, or interaction of the drug with the enzyme molecule.

Chronic hypokalemia increases (Na^+,K^+)ATPase activity, and the number of enzyme units in red blood cell membranes probably is due to the steep gradient across the cell membrane (26). It is possible that lowering the gradient by increasing total body potassium decreases (Na^+,K^+)ATPase activity. We did not observe an increase in serum potassium with canrenoate or spironolactone. Despite this observation, we tested the possibility that changes in total body potassium might cause a decline in enzyme activity by treating rats with the functionally similar diuretic, Triamterene (5 mg per kg, p.o.), and by feeding rats with a liquid diet containing

0.38 mEq per ml KCl, an amount sufficient for potassium loading (27). In these unpublished studies, serum potassium was not significantly increased by Triamterene and neither Triamterene nor potassium loading decreased hepatic (Na^+,K^+)ATPase activity (control 23.02 ± 2.0 μmoles Pi per mg protein per hr; Triamterene 28.8 ± 2.5; KCl 27.8 ± 2.2).

(Na^+,K^+)ATPase activity is so sensitive to its membrane lipid environment that Sinensky et al. proposed using this enzyme to predict altered membrane fluidity (28). Spironolactone treatment more than doubles biliary excretion of cholesterol in rats (3) suggesting that spironolactone alters hepatocyte cholesterol metabolism. Our data show that canrenoate induces a dose-dependent increase in membrane cholesterol content with concomitant decrease in membrane phospholipids. These simultaneous lipid changes amplify alterations in the cholesterol to phospholipid ratio. Furthermore, since the cholesterol to phospholipid ratio reflects membrane fluidity (29), changes in the activity of (Na^+,K^+)ATPase may be expected and demonstration of altered fluorescence polarization after canrenoate treatment is not surprising. The lipid changes in the male rat hepatocyte membrane were carefully studied after administration of the synthetic estrogen, ethinyl estradiol (5 mg per kg per day for 5 days) (30). This steroid produces changes in the cholesterol to phospholipid molar ratio (0.48 control to 0.61 treated) similar to those seen with 20 mmoles canrenoate (0.440 control to 0.569 treated; Table 3). Estrogen-induced increases in membrane cholesterol and cholesterol esters, however, were not accompanied by phospholipid changes. Inhibition of hepatic (Na^+,K^+)ATPase activity is similar in the canrenoate (55% of control after the 20-mmole dose) and estrogen [49% of control (31)]-treated animals. The nonionic detergent, Triton WR-1339, partially reversed inhibition of hepatic (Na^+,K^+)ATPase activity further paralleling the hepatic effects of canrenoate and estrogens and supporting the importance of these membrane lipid changes on hepatocyte function. Thus, the hepatocyte membrane lipid changes induced by canrenoate are similar to previously documented lipid changes due to estrogens and explain, at least in part, the decrease in (Na^+,K^+)ATPase activity. Although the membrane lipid and (Na^+,K^+)ATPase activity changes are similar after estrogens and spironolactone, marked differences are apparent in measurements of bile flow. The explanation for this discrepancy is unclear; preliminary studies suggest enhanced clearance of inulin and sucrose after spironolactone treatment implicating changes in paracellular transport of water (unpublished data).

Several possible mechanisms may account for canrenoate-induced change in membrane lipids. First, the spironolactone class of drugs acts as antialdosterone agents. Aldosterone modifies the membrane phospholipids of toad bladders by increasing oxidation of fatty acids and enhancing elongation and desaturation of oleic acid and its recycling into membrane phospholipids. The decrease in membrane phospholipids could be explained by blocking the effects of endogenous aldosterone, however, Duval et al. were unable to identify mineralocorticoid receptors in the liver (25). The administration of mineralocorticoids to intact and adrenalectomized rats does not change the activity of hepatic (Na^+,K^+)ATPase activity (32) adding indirect support to the proposal of Duval et al. However, the technical difficulties in isolating aldosterone receptors and the ambiguity of their subcellular mechanism of action (33) makes it difficult to exclude physiologic changes mediated through the aldosterone receptor.

Secondly, since glucocorticoids increase and adrenalectomy decreases (Na^+,K^+)ATPase activity (27), reduced (Na^+,K^+)ATPase activity may have resulted from decreased glucocorticoid synthesis induced by canrenoate and spironolactone. Erbler (34) demonstrated that the addition of canrenone and spironolactone to rat adrenal tissue, *in vitro*, decreased corticosteroid production. In addition, administration of spironolactone to guinea pigs produced a decline in cortisol production by adrenal slices and decreased activities of several steroidogenic enzymes including 17-α-hydroxylase, 21-hydroxylase, 11-β-hydroxylase, and cholesterol desmolase (35–37). Despite these experimental findings, serum corticosterone levels (morning values) in our animals after 20 μmoles canrenoate per 100 gm body weight per day for 3 days were nearly identical to untreated values [control (n = 12) 202 ± 25 ng per ml; canrenoate (n = 12) 206 ± 12 ng per ml, unpublished results]. These findings are in agreement with others (36) who showed no effect of spironolactone on glucocorticoids in animals whose principal glucocorticoid is corticosterone.

Thirdly, since spironolactone has estrogen-like side effects, drug interaction with specific cytosol receptors may induce subcellular lipid changes. An estrogen agonist effect could explain the similarities between the lipid changes induced by estrogens (30) and the ones noted here. Although this seems to be the most likely possibility, this hypothesis is weakened by the study of Rifka et al. (38) which postulated that estrogen-like effects of spironolactone result from inhibiting the effects of androgens since spironolactone competitively inhibits androgen binding to the dehydrotestosterone receptor and does not interact with the estrogen receptor. Current information does not permit further clarification of this possible mechanism of hepatic membrane lipid alteration by canrenoate.

Our data *in vivo* can best be explained by a metabolic effect which directly or indirectly affects the surface membrane; a nonspecific drug interaction with (Na^+,K^+)ATPase would be expected to give immediate inhibition rather than progressive inhibition noted in these experiments. The possibility of direct alteration of membrane fluidity by incorporation of the drug or a metabolite into the membrane bilayer should be considered since these compounds are lipid soluble. The *in vitro* inhibition of enzyme activity suggests direct interaction of the drug with enzyme molecules or incorporation into the membrane with secondary membrane effects on enzyme activity. It appears that lipid solubility is necessary for the *in vitro* effect, since canrenoate, the water-soluble potassium congener of canrenone, failed to inhibit (Na^+,K^+)ATPase activity *in vitro*. It is unlikely that

enzyme inhibition *in vivo* and *in vitro* is due to the same mechanism; 10 μM canrenone in the enzyme assay caused similar changes in enzyme activity in membranes isolated from untreated rats (63% of untreated control) and from rats treated with canrenoate [20 μmoles per 100 gm body weight per day × 3 days (69% of treated control)]. Triton WR-1339 also did not alter inhibition produced by *in vitro* canrenone but partially reversed inhibition in treated membranes. In addition to the attractive hypothesis of drug intercalation in the membrane, these compounds may induce inhibition *in vitro* by binding to the ouabain site on the (Na^+,K^+)ATPase molecule. Previously published work does not clarify this issue; Fricke showed lack of interaction of spironolactone with the ouabain binding site in guinea pig heart (39) while Finotti et al. (11) demonstrated competitive inhibition. Current data suggests that inhibition (Na^+,K^+)ATPase activity is mediated by different mechanisms *in vivo* and *in vitro* although direct enzyme inhibition may be responsible for a portion of the decreased (Na^+,K^+)ATPase activity. A change in the number of (Na^+,K^+)ATPase molecules may contribute to the changes in enzyme activity; the nearly complete reversal of enzyme inhibition by Triton WR-1339 suggests that a decrease in enzyme number could account for only a small portion of the inhibition.

Spironolactone and canrenone markedly decrease membrane (Na^+,K^+)ATPase activity. This change is due, at least in part, to changes in membrane lipids which decrease membrane fluidity. The changes in (Na^+,K^+)ATPase activity and membrane lipids have important experimental implications for hepatocellular transport and clinical relevance in cirrhotic patients who are treated with spironolactone.

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