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Aldosterone-Induced Membrane Phospholipid Fatty Acid Metabolism in the Toad Urinary Bladder†

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ABSTRACT: Aldosterone action in the isolated toad urinary bladder has been studied by incubation of the tissue with several specifically labeled lipogenic precursors. Within 30 min after hormone addition phospholipid synthesis is stimulated; the metabolism of oleic acid is particularly enhanced. Additionally, during this time interval a phospholipid deacylation–reacylation cycle is stimulated by aldosterone. After 4 hr aldosterone increases the oxidation of all fatty acids utilized, but enhances, specifically, the elongation and desaturation of oleic acid, as well as the recycling of [^{14}C]acetyl-CoA derived from [1-^{14}C]oleic acid into membrane phospholipid fatty acid. These data provide further evidence for a rapid and specific action of aldosterone on toad bladder membrane phospholipid fatty acid metabolism.

The addition of aldosterone to the isolated toad urinary bladder stimulates transcellular sodium transport (Sharp and Leaf, 1966; Edelman and Fimognari, 1968). Aldosterone treatment alters at least three other membrane-related processes. It increases (1) the permeability of the tissue to water and low-molecular solutes in the presence of antidiuretic hormone (Goodman et al., 1969; Handler et al., 1969); (2) the sensitivity of sodium transport to inhibition by the cardiac glycoside, ouabain, a known inhibitor of Na^+–K^+-activated ATPases (Goodman et al., 1969); and (3) the sensitivity of sodium transport to inhibition by increased partial pressures of oxygen (Allen et al., 1973). Because an alteration in membrane lipid composition could account for these diverse hormone-induced changes in tissue function, we initiated an investigation of the effects of aldosterone on membrane lipid metabolism. In our earlier studies we found that aldosterone treatment increased both lipid synthesis and the turnover of membrane phospholipid fatty acids within 30 min (Goodman et al., 1971). Additionally, pretreatment of the tissue with phospholipase A resulted in a significant reduction in the latent period between hormone addition and the inception of the physiological response, an increase in transcellular sodium transport. Aldosterone also induced an increase in the weight percentage of phospholipid long-chain polyunsaturated fatty acids. These studies thus provided the first biochemical evidence that a modification of lipid metabolism might be a significant effect of the action of aldosterone. The purpose of the present investigation was to characterize more fully and specifically the effects of aldosterone on lipid metabolism in the toad urinary bladder.

Utilizing specifically labeled [^{14}C]- and [^{3}H]fatty acids as precursors and analyzing tissue phospholipid fatty acids by radio-gas chromatography, we have shown that aldosterone stimulates specifically the elongation and desaturation of oleic acid (18:1ω9). By prelabeling tissue lipids with [^{14}C]acetate, we have also demonstrated that aldosterone stimulates the release of ^{14}C from phospholipid into [^{14}C]fatty acid.

Materials and Methods

Experimental Animals and Preincubation. Urinary bladders were removed from female toads (*Bufo marinus,* National Reagents Co., Bridgeport, Conn.) treated as previously described (Goodman et al., 1971). Tissue was routinely preincubated overnight at room temperature in substrate-free aerated Ling–Ringer phosphate buffer (Ling, 1962), pH 7.4, containing 50 mg/l. of both penicillin G and streptomycin sulfate. In experiments where tissue lipids were prelabeled, 1 m*M* sodium acetate, containing 1.0 μCi/ml of sodium [1,2-^{14}C]acetate, was included in the overnight incubation solution. The following morning experiments were performed.

Tissue Incubations; [^{14}C]Lipid Analysis. Individual matched hemibladders from six toads were incubated for 1 hr in Ling–Ringer phosphate (pH 7.4) containing 4 m*M* glucose. The tissue was then transferred into a fresh Ling–Ringer glucose buffer containing either 0.1 m*M* sodium ac-

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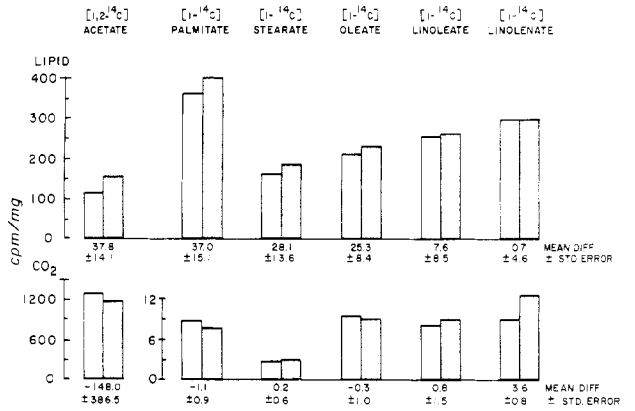

FIGURE 1: The effect of aldosterone on [^{14}C]lipid precursor incorporation into tissue lipid and oxidation during the first 30-min exposure to hormone. Matched hemibladders (six) were incubated for 30 min in the presence of specific ^{14}C-labeled substrates and either aldosterone (10^{-7} M) in methanol or carrier methanol before extraction of lipids and determination of $^{14}CO_2$. Significant increases between aldosterone and control incubations were observed for lipid incorporation in the presence of [1,2-^{14}C]acetate, [1-^{14}C]palmitate, stearate, and oleate ($P < 0.05$) and for $^{14}CO_2$ production in the presence of linolenate ($P < 0.005$).

etate (0.1 μCi/ml of sodium [1,2-^{14}C]acetate) or a [1-^{14}C]fatty acid complexed to bovine serum albumin (BSA) (0.01–0.02 μCi/ml; 0.006% BSA final concentration) (Donabedian and Karmen, 1967). One hemibladder from each toad received aldosterone (Calbiochem) 10^{-7} M in methanol, the other carrier methanol. After 30 min the tissue was quickly removed, blotted, rinsed three times in 5% BSA, and immersed in liquid N_2. The incubation medium was acidified in the presence of Hyamine hydroxide (Amersham–Searle), as previously described (Goodman et al., 1971), for determination of $^{14}CO_2$ as a measure of fatty acid oxidation. In a second series of experiments, tissue was treated with aldosterone or carrier methanol for 4 hr before transfer to a [1,2-^{14}C]acetate, a [1-^{14}C]fatty acid, or a [9,10-^{3}H]oleic acid containing buffer for a 30-min incubation. The following [1-^{14}C]fatty acids were utilized: palmitate, stearate, oleate, linoleate, and linolenate (all 55–60 mCi/mmol); all were obtained from Amersham–Searle and were analyzed for decomposition before use by radio-gas chromatography (Goodman et al., 1971). [9,10-^{3}H]Oleic acid was obtained from New England Nuclear and used at 0.03 μCi/ml–0.006% BSA final concentration. Tissue lipids were extracted from individual hemibladders as previously described (Goodman, et al., 1971), and an aliquot was utilized to determine ^{14}C incorporation into lipid. Aldosterone-treated and control samples were then pooled for analysis of the pattern of incorporation and determination of phospholipid fatty acid specific activities.

Fatty acid methyl esters were prepared according to Kishimoto and Radin (1965). Gas chromatography was carried out on a Varian Aerograph Model 2100 gas–liquid chromatograph equipped with flame ionization detectors. A 6 ft × 4 mm i.d. glass U column packed with 10% EGSS-X on 100–120 mesh Gas-Chrom P was employed for all analyses. Injector and column temperatures were maintained at 250 and 195°, respectively. Qualitative, tentative identification of the various peaks was made on the basis of (1) plots of log retention times vs. carbon number, (2) relative retention times given in the literature (Ackman and Burgher, 1963), and (3) chromatography with esters of known structure. To determine the specific activity of fatty

Table I: Pattern of Labeling of Toad Bladder Lipid in the Presence of ^{14}C-Labeled Precursor.[a]

Lipid	% of Total [^{14}C] Lipid Labeled	
	[1,2-^{14}C] Acetate	[1-^{14}C] Palmitate
P[b]	56.2	84.6
MG	11.0	4.7
FA	3.3	0.1
CHOL	2.3	1.3
DG	5.6	2.9
TG	5.8	2.3
CE	13.9	4.2

[a] Matched hemibladders were incubated for 30 min in the presence of a [^{14}C]lipid precursor either with or without aldosterone (10^{-7} M) before extraction and thin-layer chromatographic separation of the [^{14}C]lipid. Data from control tissue only are shown since there were no differences in the pattern of labeling in the presence of any of the substrates utilized between control and aldosterone-treated tissue. [b] Abbreviations used are: P, phospholipids; MG, monoglycerides; FA, fatty acids; CHOL, cholesterol; DG, diglycerides; TG, triglycerides; CE, cholesterol esters.

acids, radioactive fatty acid methyl esters derived from tissue lipids incubated in the presence of either ^{14}C- or ^{3}H-labeled substrate were collected utilizing a Varian Model 96-0000-18 10:1 glass splitter–collector.

Hemibladders incubated overnight in [1,2-^{14}C]acetate were transferred to fresh Ling–Ringer glucose buffer containing 1 mM sodium acetate, incubated for 15 min, and then transferred again to a fresh Ling–Ringer glucose buffer containing either aldosterone or carrier methanol. After 30 min the tissue was frozen in liquid N_2 and the $^{14}CO_2$ produced determined as described above. Tissue lipids were extracted and the specific activities of cellular free fatty acids and phospholipid fatty acids were determined after separation of these lipid classes. Silica gel G column chromatography was used to separate neutral and phospholipids (Goodman et al., 1971); free fatty acids were removed from the neutral lipids by sodium carbonate extraction (Dittmer and Wells, 1969).

Results

In our initial studies of lipid metabolism in the toad urinary bladder we demonstrated that aldosterone stimulated the conversion of [2-^{14}C]pyruvate to lipid and increased the specific activity of phospholipid fatty acids. To further elucidate these effects we have employed additional ^{14}C-labeled lipogenic substrates. As shown in Figure 1, within 30 min after its addition aldosterone caused a small but significant ($P < 0.05$) increase in the conversion of ^{14}C-labeled medium acetate, palmitate (16:0), stearate (18:0), and oleate (18:1ω9) to lipid; no increase in the conversion of linoleate (18:2ω6) or linolenate (18:3ω3) to lipid was observed. During this initial time interval the oxidation of only linolenate was stimulated by aldosterone.

Utilizing [1,2-^{14}C]acetate as lipogenic precursor, 55–60% of the incorporated ^{14}C-labeled product was recovered as [^{14}C]phospholipid; in the presence of each of the [^{14}C]fatty acids phospholipid accounted for 85–90% of the [^{14}C]lipid product. Aldosterone pretreatment did not alter this pattern of incorporation. Table I, therefore, consists of data derived from control tissue incubated in the presence of [1,2-^{14}C]acetate and [1-^{14}C]palmitate, taken as a representative [1-^{14}C]fatty acid precursor. Additionally, aldosterone did not change the pattern of distribution of any of the ^{14}C-labeled substrates into the various specific cellular

Table II: Pattern of Toad Bladder Phospholipid Labeling in the Presence of a ^{14}C-Labeled Precursor.[a]

Lipid	% of Total [^{14}C] Phospholipid Labeled					
	[1,2-^{14}C] Acetate	[1-^{14}C] Palmitate	[1-^{14}C] Stearate	[1-^{14}C] Oleate	[1-^{14}C] Linolate	[1-^{14}C] Linolenate
LPC[b]	2.2	2.1	0.3	0.5	0.3	0.3
SPH	1.8	1.8	1.1	0.8	0.4	0.4
PS	8.4	4.1	22.1	4.6	1.2	0.8
PI	9.8	6.9	27.4	6.2	8.6	6.2
PC	53.5	77.7	31.3	45.9	69.0	73.1
PE	23.1	5.3	17.1	41.2	17.2	18.9
CL	1.3	2.3	0.3	0.8	0.1	0.2

[a] Matched hemibladders were incubated for 30 min in the presence of a [^{14}C] lipid precursor either with or without aldosterone (10^{-7} *M*) before extraction and thin-layer chromatographic separation of [^{14}C] phospholipid. Only data from control tissue are shown since there were no differences in the pattern of labeling in the presence of any of the substrates utilized between control and aldesterone-treated tissue. [b] Abbreviations used are: LPC, lysophosphatidylcholine; SPH, sphingomyelin; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; CL, cardiolipin.

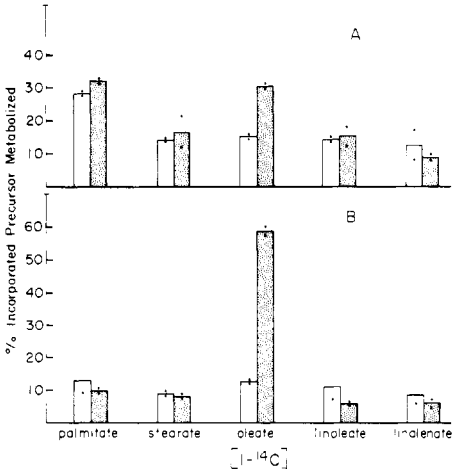

FIGURE 2: The effect of aldosterone on the metabolism of specific [1-^{14}C]fatty acids incorporated into phospholipid after 30 min (A) or 4 hr (B) exposure to hormone. Fatty acid methyl esters prepared from the phospholipids of the tissues were analyzed by radio-gas chromatography. The data are represented as the percentage of the total incorporated radioactivity recovered with retention time different than the specific precursor [1-^{14}C]fatty acid employed. The results are represented as the mean of two separate determinations.

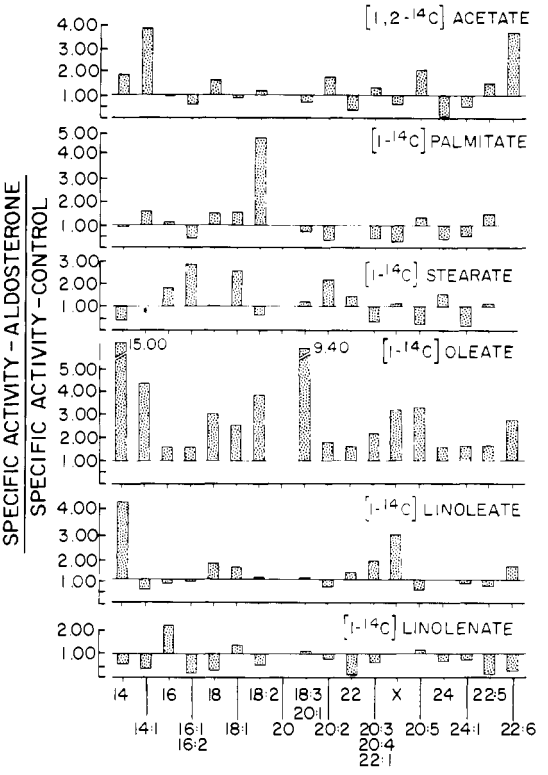

FIGURE 3: Relative specific activities of ^{14}C-labeled phospholipid fatty acids derived from toad bladder after 30-min aldosterone treatment. Each set of data is derived from tissue incubated in the presence of a specific ^{14}C-labeled lipogenic precursor. The specific activity of each individual fatty acid class was calculated by dividing the amount of radioactivity recovered by the mass response of the detector. The symbol * indicates that significant radioactivity was recovered only in extracts from control tissue. No bar over a particular fatty acid class indicates that no significant radioactivity was recovered in extracts prepared from either control or hormone-treated tissue. The results for each lipogenic precursor are represented as the mean of two separate determinations.

phospholipids. Table II shows data derived from control tissue only. Most notable, when all ^{14}C precursors are compared, is the very large proportion of [1-^{14}C]oleate incorporated into phosphatidylethanolamine relative to the proportion incorporated into phosphatidylcholine and the marked extent of incorporation of [1-^{14}C]stearate into phosphatidylserine and phosphatidylinositol.

Examination of the [^{14}C]phospholipid fatty acids by radio-gas chromatography, however, revealed a striking effect of aldosterone; the steroid stimulated the metabolism of specific [1-^{14}C]fatty acids (Figure 2A). The rate of conversion of [1-^{14}C]oleate to other [^{14}C]fatty acids in phospholipids was doubled after a 30-min exposure to aldosterone, palmitate and stearate conversions were stimulated only to a slight extent, linoleate conversion was not altered, and linolenate conversion inhibited slightly. Analysis of the specific activity of individual phospholipid fatty acid classes revealed a highly specific effect of aldosterone (Figure 3). With [1,2-^{14}C]acetate as substrate, aldosterone treatment resulted in an increased specific activity of several fatty acid classes. Most prominent increases were seen in 14:1, 20:5, and 22:6. These increases were not generalized as several phospholipid fatty acid classes derived from aldosterone-treated tissue showed a decrease in specific activity, e.g., (24:0). When [1-^{14}C]palmitate was the fatty acid precursor, aldosterone treatment resulted in a large increase only in the specific activity of 18:2 and a generalized decrease in the specific activity of long-chain polyunsaturated phospholipid fatty acids, except for 20:5 and 22:5 whose specific activities showed a 40–50% increase. Aldosterone produced prominent increases in the specific activity of 18:1 and 20:2 when [1-^{14}C] stearate was precursor. The changes in specific activity of fatty acid classes shorter than stearate pro-

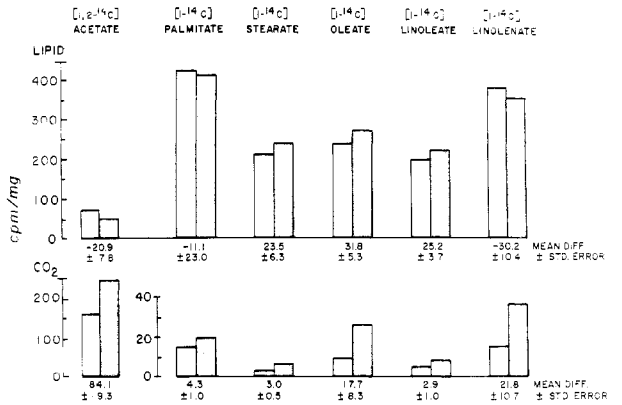

FIGURE 4: The effect of aldosterone on [^{14}C]lipid precursor incorporation into tissue lipid and oxidation after a 4-hr exposure to hormone. Matched hemibladders (six) were incubated for 4 hr in the presence of either aldosterone (10^{-7} M) in methanol or carrier methanol, and then exposed to [^{14}C]lipid precursor for 30 min before extraction of tissue lipids and determination of $^{14}CO_2$ production. Significant increases between aldosterone and control incubations were observed for lipid incorporation in the presence of [1-^{14}C]stearate ($P < 0.01$), [1-^{14}C]oleate ($P < 0.0025$), and linoleate ($P < 0.0025$); significant inhibition was observed in the presence of [1,2-^{14}C]acetate and [1-^{14}C]linolenate ($P < 0.025$). Significant differences in substrate oxidation were observed in the presence of all substrates: [1,2-^{14}C]acetate ($P < 0.005$), [1-^{14}C]palmitate ($P < 0.005$), [1-^{14}C]stearate ($P < 0.0025$), [1-^{14}C]oleate ($P < 0.05$), [1-^{14}C]linoleate ($P < 0.025$), and [1-^{14}C]linolenate ($P < 0.05$).

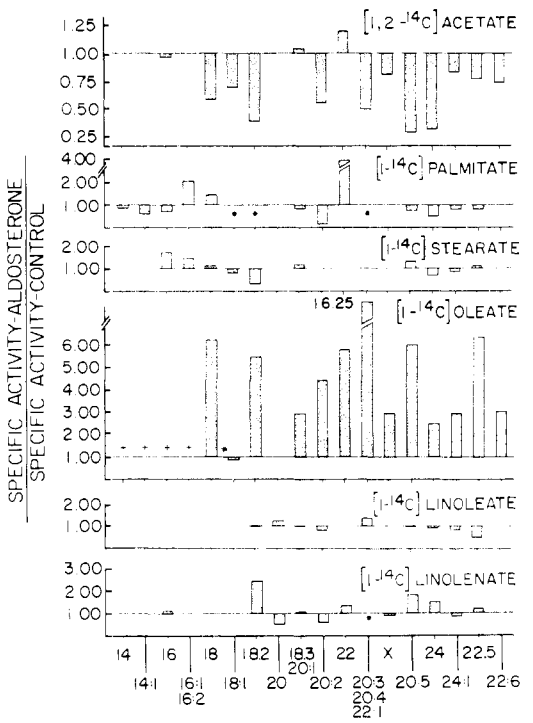

FIGURE 5: Relative specific activities of 1-^{14}C-labeled phospholipid fatty acids derived from toad bladder exposed to aldosterone for 4 hr and then for 30 min to ^{14}C-labeled lipogenic precursor. The data are represented as described in Figure 3. The symbol + indicates that significant radioactivity was recovered only in extracts from aldosterone-treated tissue.

duced by aldosterone probably reflect hormone-induced alterations in recycling of [^{14}C]acetyl-CoA derived from oxidized [1-^{14}C]fatty acid substrate.

The largest changes in phospholipid fatty acid specific activities induced by aldosterone occurred when [1-^{14}C]oleate was precursor. A generalized increase in all classes was observed; the largest increase (ninefold) was observed in a mixed peak containing 20:1 and 18:3; a two- to fourfold increase occurred in several other classes as well. Fifteenfold and fourfold increases are observed in 14 and 14:1, respectively. As in the case of [1-^{14}C]stearate, these changes probably reflect recycling of [^{14}C]acetyl-CoA derived from catabolism of labeled oleate. In the presence of [1-^{14}C]linoleate and [1-^{14}C]linolenate, aldosterone induced large increases in the specific activity of short-chain fatty acids. In general, with [1-^{14}C]linoleate there were no striking increases except for 14:0 and an unidentified class with retention just greater than arachidonate (20:4ω6). In the presence of [1-^{14}C]linolenate all fatty acid classes showed a decrease in specific acitivity except 16:0 and 18:1. These very striking, rapid effects of aldosterone on fatty acid metabolism could result from hormone-induced changes in the specific activity of intracellular precursor fatty acyl-CoA pools. Such changes could result from changes in intracellular free fatty acid levels. This was not the case, however, since aldosterone produced no changes in tissue free fatty acid composition.

These results were derived from tissue exposed to aldosterone and ^{14}C-labeled substrate during the initial 30 min of the incubation. During this time interval there was no observable increase in sodium transport induced by aldosterone. In a second series of experiments, tissues were exposed to aldosterone for 4 hr before a 30-min pulse with ^{14}C-labeled substrate. After 4-hr exposure to aldosterone, [1,2-^{14}C]acetate and [1-^{14}C]linolenate incorporation into tissue lipid was decreased when compared with matched control tissue, but incorporation of [1-^{14}C]stearate, [1-^{14}C]oleate, and [1-^{14}C]linoleate was increased (Figure 4). Incorporation of [1-^{14}C]palmitate was not altered significantly by aldosterone. The oxidation of all substrates employed was increased after this prolonged exposure to aldosterone. As in the previous series of experiments (Tables I and II), aldosterone did not alter the pattern of lipid incorporation into principal lipid classes or into specific phospholipid classes. The data for this series of experiments are not shown since they are not significantly different from the data shown in Tables I and II for tissue lipid distribution after short exposure to aldosterone.

When the fate of the ^{14}C-labeled substrates in these longer term studies was examined by radio–gas chromatography, the effect of aldosterone again appeared highly specific (Figure 2B). The conversion of [1-^{14}C]oleate to other fatty acids was enhanced approximately fourfold, but the metabolism of the other ^{14}C-labeled substrate was either not altered ([1-^{14}C]stearate) or inhibited ([1-^{14}C]palmitate, [1-^{14}C]linoleate, and [1-^{14}C]linolenate). Detailed analysis of individual phospholipid fatty acid specific activities further demonstrated a generalized decrease in specific activity induced by aldosterone in the presence of [1,2-^{14}C]acetate (Figure 5). In the presence of [1-^{14}C]palmitate, aldosterone induced an increase in the specific activity of 16:1, 2, 18:0, and 22:0, and a decrease in all other classes. In the presence of [1-^{14}C]stearate, [1-^{14}C]linoleate, and [1-^{14}C]linolenate, hormone treatment produced an alteration in the specific activity of a number of individual phospholipid fatty acids, but these changes were not as prominent as those produced by aldosterone when [1-^{14}C]oleate served as precursor. After 4 hr of aldosterone treatment a generalized (3- to 16-fold) increase in fatty acid specific activity was seen when [1-^{14}C]oleate served as precursor. To differentiate between [^{14}C]fatty acid arising from direct elongation and desatura-

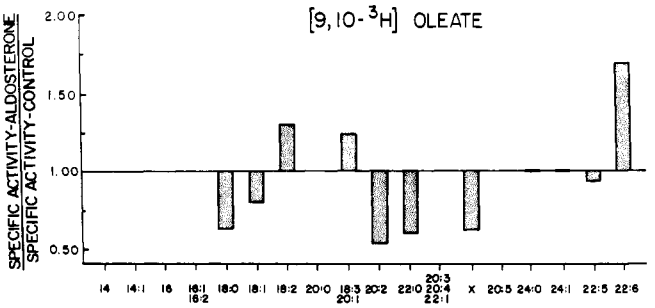

FIGURE 6: Relative specific activities of [9,10-^{3}H]oleic acid labeled phospholipid fatty acids derived from toad bladder exposed to aldosterone for 4 hr and then for 30 min to [9,10-^{3}H]oleic acid. The data are represented as in Figures 3 and 5.

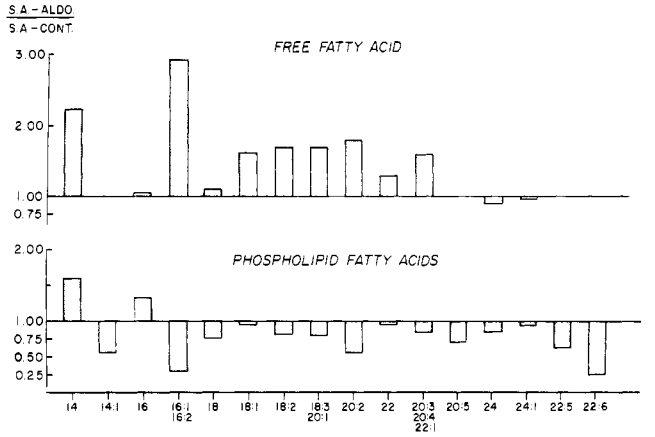

FIGURE 7: The effect of aldosterone on endogenous toad bladder phospholipase activity. Matched hemibladders were incubated overnight in [1,2-^{14}C]acetate and then placed in fresh nonradioactive buffer and exposed to aldosterone (10^{-7} M) in methanol or carrier methanol for 30 min. Radio-gas chromatography of the fatty acid methyl esters prepared from the free fatty acids and phospholipids of lipid extracts of these tissues was performed to determine the relative specific activities of the constituent fatty acids. The results are represented as the mean of two separate determinations.

tion of [1-^{14}C]oleate and [^{14}C]fatty acid arising from recycled [^{14}C]acetyl-CoA derived from the catabolism of [1-^{14}C]oleate, [9,10-^{3}H]oleate was used as precursor. Analysis of phospholipid fatty acid specific activities after 4-hr exposure to aldosterone and a 30-min pulse with [^{3}H]fatty acid again demonstrated an effect of aldosterone on oleate desaturation and elongation (Figure 6). The specific activity of 18:2, a mixed peak containing 18:3 and 20:1 and 22:6, was increased by hormone treatment with a concomitant fall in the specific activity of several other fatty acid classes. Both 18:2 and 18:3 are products of oleate (18:1) desaturation, while 20:1 is a product of oleate elongation. Additionally, these data, taken together with the data on the effect of aldosterone in the presence of [1-^{14}C]oleate (Figure 5), also indicate a striking stimulatory effect of aldosterone on the recycling of [^{14}C]acetyl-CoA derived from precursor [1-^{14}C]oleic acid.

To explore the possibility that aldosterone might alter the deacylation of preformed tissue phospholipids, tissue lipids were labeled by overnight incubation with [1,2-^{14}C]acetate. After washing in a nonradioactive, acetate-containing buffer, tissue was incubated with aldosterone for 30 min. Tissue lipids were then extracted and the specific activities of phospholipid fatty acids and free fatty acids determined. As stated earlier, aldosterone treatment did not alter the free fatty acid composition of the toad bladder or the oxidation of prelabeled tissue lipid (data not shown), but produced a generalized increase in the specific activity of free fatty acids in the tissue (Figure 7). Associated with this increase in free fatty acid specific activity was a generalized fall in phospholipid fatty acid specific activity.

Discussion

Previous biochemical studies of aldosterone action in the toad urinary bladder have emphasized the possible role of hormone-induced gene activation and enhanced synthesis of specific proteins (Edelman and Fimognari, 1968). These studies, however, have usually employed prolonged hormone incubations (greater than the 45–90-min latent period). As yet, no detailed description of a specific aldosterone-induced ribonucleic acid(s) or protein(s) has appeared although several preliminary reports (Benjamin and Singer, 1974; Rossier et al., 1974) have been published. Since aldosterone alters several membrane functions in the toad bladder (see above) and lipid composition of membranes is a key factor in membrane structure and function (Linden et al., 1973; Machtiger and Fox, 1973; Horwitz et al., 1974; Silbert et al., 1974), we have been investigating hormone-induced changes in membrane phospholipid metabolism. Previously, we had shown that within 30 min after aldosterone addition there is a stimulation of both fatty acid synthesis and the turnover of membrane phospholipid fatty acids (Goodman et al., 1971). After 6 hr the weight percentage of membrane phospholipid polyunsaturated fatty acids was increased by aldosterone. The present investigation provides further biochemical evidence for rapid effects of aldosterone on toad bladder lipid metabolism and delineates the specificity of these effects.

By utilizing [1,2-^{14}C]acetate and a number of [1-^{14}C]fatty acids, several specific effects of aldosterone have been observed. The incorporation of acetate, palmitate, stearate, and oleate into tissue phospholipid is stimulated within the first 30 min after hormone treatment. Additionally, the oxidation of [1-^{14}C]linolenate, but none of the other ^{14}C-labeled substrates employed, is increased by aldosterone during this time interval (Figure 1). These data suggest, but do not prove conclusively, that aldosterone is exerting its effect(s) on lipid metabolism at a step or steps subsequent to fatty acid uptake and not on uptake itself.

When aldosterone is added for 30 min to tissue whose lipids have been prelabeled with [^{14}C]acetate, tissue-free fatty acid specific activities rise and phospholipid fatty acid specific activities fall (Figure 7). During this incubation there is no measurable increase in the oxidation of [^{14}C]lipid. These data are consistent with a stimulation of an endogenous phospholipase activity during this early time period, the latent period between hormone addition, and the increase in short-circuit current. These data, however, do not exclude the possibility that aldosterone might be altering the compartmentation or reutilization of [^{14}C]lipid. Taken together with our previous data, these results suggest that aldosterone might stimulate the deacylation and reacylation of membrane phospholipids before any hormone-induced increase in sodium transport is observed.

Although aldosterone stimulated the conversion of several [^{14}C]fatty acid classes into membrane phospholipid, analysis of the specific activity of phospholipid fatty acids demonstrated further specificity of the alteration in lipid metabolism induced by aldosterone (Figures 2 and 3). In the presence of [1,2-^{14}C]acetate the specific activity of several classes was increased. Most prominent, however, was

the doubling in the rate of oleic acid metabolism and the generalized increase in the specific activity of all phospholipid fatty acids in the presence of [1-^{14}C]oleate.

After 4-hr exposure to aldosterone the oxidation of all ^{14}C-labeled substrates employed was increased (Figure 4). This generalized increase in oxidative metabolism may reflect the increased cellular ATP requirements needed to sustain the aldosterone-induced increase in active transepithelial sodium transport. In contrast to this generalized effect of aldosterone on substrate oxidation, aldosterone had an even more specific effect on cellular lipid metabolism. After prolonged incubation (4 hr) aldosterone stimulated the conversion of stearate, oleate, and linoleate and inhibited conversion of acetate and linolenate to phospholipid fatty acid. No change in palmitate conversion to phospholipid fatty acid was observed. Analysis of the extent of metabolism of each fatty acid to other fatty acid classes again demonstrated that aldosterone exerted a specific effect on oleate metabolism (Figure 2B). After prolonged exposure to hormone, oleic acid conversion was fourfold greater than in control tissue. This contrasts with the twofold stimulation of this same parameter of lipid metabolism observed in the first 30 min after aldosterone treatment. When individual fatty acid specific activities were compared (Figure 5) it was clear that aldosterone treatment led to a generalized increase in phospholipid fatty acid specific activities when [1-^{14}C]oleic acid served as radioactive precursor. The results obtained, utilizing [9,10-^{3}H]oleic acid under the same experimental conditions (Figure 7), indicate that aldosterone does indeed promote the elongation and desaturation of oleic acid. However, the observed generalized increase in the specific activities of phospholipid fatty acids must be, in large part, due to the reincorporation of [^{14}C]acetyl-CoA derived from precursor [1-^{14}C]oleate. It would be extremely informative to be able to quantitate more precisely in the intact tissue the magnitude of oleate elongation and desaturation as compared to the extent of the recycling of the terminal acetyl-CoA derived from the original [1-^{14}C]oleate. This would require determination of the specific activities of the fatty acyl-CoA's as well as quantitative oxidative decarboxylation (Brady et al., 1960) of the individually isolated phospholipid fatty acids and fatty acyl-CoA's. This procedure is, however, technically not feasible because of the quantities of materials required.

In the presence of the other [1-^{14}C]fatty acids, prominent increases were not observed in fatty acid specific activities after aldosterone treatment. In general, individual specific activities were not changed or decreased. In the presence of [1,2-^{14}C]acetate, aldosterone treatment produced a striking decrease in the specific activities of phospholipid fatty acids. This reflects preferential utilization of the [^{14}C]acetyl-CoA formed from the added [1,2-^{14}C]acetate for oxidation rather than for lipogenesis, a result consistent with the toad bladder's increased utilization of ATP to support increased active transcellular sodium transport.

The results discussed above indicate that aldosterone exerts selective effects on oleic acid metabolism. Aldosterone stimulates oleate desaturation and elongation (Figure 7). In addition, aldosterone stimulates extensive recycling of [^{14}C]acetyl-CoA derived from [1-^{14}C]oleate. This [^{14}C]acetyl-CoA does not appear to be derived from mitochondrial β oxidation since aldosterone stimulates a generalized increase in phospholipid fatty acid specific activities (Figure 3) without altering [1-^{14}C]oleate oxidation (Figure 1) during the first 30 min exposure to hormone. The subcellular localization of this phase of aldosterone action cannot be ascertained from the current experiments, and there is no reported work on fatty acid metabolism from other biological systems which illuminates this specific problem. Additionally, it is quite clear that under our experimental condition the acetyl-CoA derived from β oxidation of fatty acids is not in equilibrium with the acetyl-CoA pool utilized for fatty acid biosynthesis and elongation. Aldosterone stimulates [1-^{14}C]linolenate oxidation during the latent period (Figure 1) but does not cause prominent increases in phospholipid fatty acid specific activities (Figure 3) in the presence of this [1-^{14}C]-labeled precursor. After 4 hr exposure to hormone the oxidation of all substrates is increased (Figure 4).

If the acetyl-CoA pool utilized for lipid synthesis were in equilibrium with the acetyl-CoA derived from β oxidation of fatty acids, then increased phospholipid fatty acid specific activities should be observed after hormone treatment in the presence of each of the [1-^{14}C]fatty acids utilized. This clearly is not the case, i.e. see Figure 6, especially linoleate. In rat liver, where detailed studies of this problem of intracellular compartmentation have been carried out (Dietschy and McGarry, 1974), it has also been concluded that the mitochondrial and cytoplasmic acetyl-CoA pools are not in equilibrium. If the same is true in the toad bladder, our data suggest that aldosterone stimulates, specifically, the cytosolic or microsomal oxidation of oleate but not other fatty acids, and that the acetyl-CoA derived from this oxidation is utilized preferentially for chain elongation.

The current data point to a rapid effect of aldosterone on mitochondria (fatty acid oxidation), the soluble cytoplasmic fatty acid synthetase, and the endoplasmic reticulum (fatty elongation and desaturation and acyl transferase). Whether these hormone-induced alterations in fatty acid metabolism are secondary to some nuclear event(s) cannot be ascertained from the present results. Studies of the mechanism of action of several steroid hormones have led to the concept that the particular hormone first complexes with a cytoplasmic receptor protein before it is transported into the nucleus (Jensen and DeSombre, 1972). In the nucleus the hormone–receptor complex interacts with the cellular genome. In the toad bladder aldosterone binding has been demonstrated in a nuclear fraction (Ausiello and Sharp, 1968). The precise relationship between the association of aldosterone with this subcellular fraction and the biochemical data presented in the present study is, however, difficult to reconcile. Additionally, it is not clear which subcellular membrane(s') deacylation (Figure 7) might be stimulated by aldosterone addition. Phospholipase activity has been described in crude toad bladder homogenates (Rosenbloom and Elsbach, 1968), but subcellular localization of this activity has not been reported. The present data demonstrate that aldosterone evokes a rapid increase in phospholipid fatty acid turnover. This may reflect the stimulation of a deacylation–reacylation cycle, and it raises the possibility that aldosterone's effect on membrane function could be due to altered membrane lipid composition.

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Asymmetric Exchange of Vesicle Phospholipids Catalyzed by the Phosphatidylcholine Exchange Protein. Measurement of Inside–Outside Transitions†

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ABSTRACT: Purified phosphatidylcholine exchange protein was used to exchange phosphatidylcholine between homogeneous single-walled phosphatidylcholine vesicles and human erythrocyte ghosts. When excess ghosts were present, it was found that only 70% of the vesicle phosphatidylcholine was available for exchange. This fraction corresponds closely to the amount of phosphatidylcholine in the outer monolayer of these vesicles, indicating that only the outer surface of the vesicle is accessible to the exchange protein. Also, it was found that all phosphatidylcholine introduced into vesicles by the exchange protein was available for subsequent exchange. Using the exchange protein, asymmetrical vesicles were prepared in which the outer monolayer was either enriched or depleted in radioactive phosphatidylcholine as compared to the inner monolayer. Reequilibration of the radioactivity between the two surfaces of the vesicle (flip-flop) could not be detected, even after 5 days at 37°. It is estimated that the half-time for flip-flop is in excess of 11 days at 37°. These results indicate that the properties of the exchange protein can be exploited to measure phosphatidylcholine flip-flop rates and possible phosphatidylcholine asymmetry in biological and model membranes, without altering the structure of the membrane.

The exchange of phospholipids between membranes is catalyzed by soluble proteins which occur in several tissues (Wirtz and Zilversmit, 1968, 1969; Zilversmit, 1971; Wirtz et al., 1972; Enholm and Zilversmit, 1973). Of these, a phosphatidylcholine exchange protein from liver has been studied in the most detail. This protein, which has been purified to homogeneity (Kamp et al., 1973), is capable of exchanging PC[1] between a variety of different PC containing membranes (Wirtz and Zilversmit, 1968; Zilversmit, 1971; Wirtz et al., 1972) with absolute specificity (Wirtz et al., 1972; Kamp et al., 1973; Harvey et al., 1973) and with no net transfer of PC (Wirtz and Zilversmit, 1968). It acts by equilibrating the PC among the substrate membranes (Demel et al., 1973). In order to investigate whether this equilibrium involves all the PC in the membrane, or merely that fraction present on the same side of the membrane as the exchange protein, we have undertaken a detailed investigation of the kinetics of the exchange reaction using a model membrane of known structure as a substrate. In the process, we have employed the properties of the exchange protein to measure the rate of transbilayer movement of PC in vesicles, the so-called "flip-flop" process. A preliminary report of this work has been published elsewhere (Dawidowicz and Rothman, 1975).

Materials and Methods

Isotopes. [*methyl*-^{3}H]Choline chloride (2.34 Ci/mmol),

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[1] Abbreviations used are: PC, phosphatidylcholine; DOPC, 1,2-dioleoylphosphatidylcholine; KCl-Tris buffer, 0.1 *M* KCl–0.01 *M* 2-mercaptoethanol–0.01 *M* Tris-HCl (pH 7.4).