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Studies on the Mechanism of Action of Aldosterone: Hormone-Induced Changes in Lipid Metabolism*

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ABSTRACT: Studies to elucidate the mode of action of aldosterone in the amphibian urinary bladder have been carried out. Within 20 min after its addition, aldosterone causes increased decarboxylation of [1-^{14}C]glucose and an alteration in the pattern of conversion of uniformly labeled [^{14}C]glucose into lipid, suggesting stimulation of the hexose monophosphate shunt and increased lipid synthesis. With [2-^{14}C]pyruvate as radioactive precursor aldosterone stimulates lipid labeling and increased incorporation of radioactive label into the 2 position of phospholipids during a 60-min incubation. No specific individual phospholipid class was preferentially labeled. Gas–liquid chromatography of the [^{14}C]fatty acids derived from phospholipids from toad bladders treated with aldosterone for 30 and 90 min revealed an increase in the specific activity of fatty acids of chain length up to 18:2. After 6 hr, phospholipid fatty acids from hormone treated tissue show an increase in the weight percentage and specific activity of several long-chain polyunsaturated fatty acids. In addition, pretreatment of bladders with a phospholipase A_2 containing solution significantly shortened the "latent period" observed before the aldosterone-induced increase in sodium transport. These results suggest that a fundamental action of aldosterone in the toad urinary bladder is to alter the fatty acid metabolism of membrane phospholipids.

The toad urinary bladder has been used as an *in vitro* system to elucidate the mode of action of the steroid hormone aldosterone (Sharp and Leaf, 1966; Edelman and Fimognari, 1968). Addition of aldosterone leads to an increase in the active transepithelial transport of sodium after a latent period of 45–120 min. Because the observed augmentation of sodium transport is prevented by pretreatment with actinomycin D or puromycin, the hormone has been thought to act *via* the synthesis of new RNA and protein molecules (Edelman *et al.*, 1963; Porter *et al.*, 1964). Two proposals have been advanced to explain the function of the hormonally induced protein(s): (1) the hormone increases sodium permeation across the mucosal cell surface; (2) the hormone increases the supply of energy for the active extrusion of sodium across the serosal cell surface (Sharp and Leaf, 1966; Edelman, 1968).

Our interest in the problem of aldosterone action arose from the results of an investigation of the effects of hyperbaric oxygen on toad bladder function (Allen and Rasmussen, 1971). In this study, it was observed that under appropriate metabolic conditions, high partial pressures of atmospheric oxygen led to a reversible inhibition of transcellular sodium transport. This inhibition was greatly potentiated by prior treatment of the tissue with aldosterone. Since the intracellular level of ATP rose, this indicated that the inhibition of transport resulted from either an inhibition of Na^+–K^+-activated ATPase or an inhibition of Na^+ entry. Work in other systems (O'Malley *et al.*, 1966; Schauenstein, 1967; Haugard, 1968) had led to the concept that lipid peroxide formation may be an important aspect of the biochemical events underlying oxygen toxicity. It therefore seemed possible that the potentiation of poisoning by aldosterone in the toad bladder might be due to an alteration in the lipid composition of the bladder cell membranes. In addition, aldosterone treatment causes an alteration of two other membrane-related processes. Goodman *et al.* (1969) found that pretreatment of the bladder with aldosterone led to the potentiation of the action of the peptide

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hormone vasopressin upon both sodium transport and bulk water flow across this membrane. Similar findings have also been reported by Handler *et al.* (1969a). Finally, pretreatment of this tissue with aldosterone led to an increased sensitivity of sodium transport to inhibition by the cardiac gylcoside, ouabain, known to bind to and inhibit membrane-bound Na^+–K^+-activated ATPases. Thus, three different lines of evidence suggested that aldosterone might act by changing the lipid composition of the membranes of the toad bladder.

To test this hypothesis, we undertook an investigation of the effects of aldosterone upon lipid metabolism in this tissue. The present results show that aldosterone alters lipid metabolism in the toad bladder in three ways: by increasing (1) the conversion of [2-^{14}C]pyruvate into fatty acids, (2) the incorporation of newly synthesized lipid into the 2 position of phospholipids, and (3) the weight percentage of several long-chain polyunsaturated fatty acids in the phospholipid fraction of the tissue lipids.

Materials and Methods

Experimental Animals and Preincubation. Female toads (*Bufo marinus*) were obtained from National Reagents Co., Bridgeport, Conn., and kept on moist bedding at 70 ± 5°F. To reduce endogenous aldosterone secretion, all animals were placed in 0.1 M NaCl for 4–6 hr prior to an experiment. Animals were then quickly pithed and their two hemibladders removed and placed overnight at room temperature in a substrate-free aerated Ling–Ringer phosphate solution (Ling, 1962), pH 7.4, that contained 50 mg/l. of both penicillin G and streptomycin sulfate. The following morning experiments were performed.

Substrate Decarboxylations. The hemibladders from four to six toads were each cut into approximately four equal pieces and distributed into erlenmeyer flasks containing Ling–Ringer phosphate (pH 7.4) with 4 mM glucose. The tissue was incubated with gentle shaking for 1 hr at room temperature and then changed into 25 ml of fresh substrate-containing buffer. These flasks contained, in addition, 0.1 μCi/ml of either [1-^{14}C]glucose or [6-^{14}C]glucose. The incubation was continued with the flasks open to the air. At appropriate times, 1-ml aliquots were taken from each flask and the [^{14}C]CO_2 content of the sample determined by conventional techniques in closed erlenmeyer flasks with center walls containing Hyamine hydroxide (Nuclear-Chicago). A Packard liquid scintillation counter Model 3001 was used for this and all other radioactivity determinations. This open-flask incubation technique was devised because of the inherent variability in substrate decarboxylation encountered from one animal to another. Utilizing this technique the same pieces of tissue could be monitored throughout the entire incubation and each incubation flask served as its own control. No measurable loss of [^{14}C]CO_2 to the atmosphere occurred with this open-flask technique as the same pieces of tissues exhibited equal rates of substrate decarboxylation when studied in control experiments in either open flasks or the conventional closed flasks. At the appropriate time, either aldosterone (Calbiochem) 10^{-7} M in methanol or carrier methanol was added to each flask. All radioactive compounds used in this study were purchased from New England Nuclear.

Incubation and Lipid Extraction. The epithelial cells from 15 to 20 toad bladders were scraped from their connective tissue backing into ice-chilled Ling–Ringer phosphate containing 4 mM glucose, washed three times quickly, and divided approximately equally into two flasks containing fresh buffer enriched with substrate and 0.1 μCi/ml of [U-^{14}C]glucose. The incubation was carried out for 1 hr, after which aldosterone 10^{-7} M or carrier methanol was added to the appropriate flasks and the experiment allowed to continue for 20 or 90 min. The cells were then quickly decanted into chilled centrifuge tubes, which were subsequently spun for 1 min at top speed on an International Equipment Co. Model CL clinical centrifuge. The cells were quickly resuspended and washed three times before being extracted with 15 ml of chloroform–methanol (2:1, v/v). The extraction tubes were gassed with nitrogen and tightly sealed, and the lipid extraction allowed to continue overnight at room temperature in a rotary shaker. The extracts were then filtered through glass wool, washed three times with two-tenths volume of 0.1% NaCl, and concentrated under a gentle stream of nitrogen before further analysis. For quantitative studies of substrate incorporation into lipids, and characterization studies of labeled lipids, matched, intact hemibladders from four toads were used in each experiment. The preincubation in radioactive substrate before hormone addition was similar to that described above except that a combination of 4 mM glucose and 0.5 mM sodium pyruvate was used as substrate with 0.1 μCi/ml of [2-^{14}C]-pyruvate as label. Bladders were exposed to aldosterone for 1 hr, blotted gently on filter paper, and dropped into liquid nitrogen. The frozen tissue was then pulverized to a fine powder with a stainless steel mortar and pestle, kept in Dry Ice. The frozen powder was then placed in chloroform–methanol (2:1, v/v), homogenized quickly, and extracted as described above. [^{3}H]Palmitic acid was added to the extract before washing to act as a standard for correction of lipid recovery during the washing. The weight of the lipid in the extract was determined by weighing dried aliquots of the extract. Protein concentration was determined by the method of Lowry *et al.* (1951).

Lipid Fractionation and Determination of Pattern of Incorporation. The pattern of neutral lipid labeling after hormone treatment was determined utilizing the Freeman and West (1966) double development thin-layer chromatographic procedure for separating principal lipid classes. To determine the pattern of phospholipid labeling, neutral lipids and phospholipids were first separated by absorption of the total lipid extract onto silica gel G and elution of neutral lipids with solvent system I of Freeman and West (diethyl ether–benzene–ethanol–acetic acid, 40:50:2:0.2, v/v). Phospholipids were then eluted with methanol and concentrated under N_2. The phospholipid classes were then separated on a thin layer of silica gel H using a chloroform–methanol–acetic acid water (50:30:8:4, v/v) solvent system (Skipski *et al.*, 1964). In all experiments chromatographic lipid standards (Applied Science Laboratories, and Sigma Chemical Co.) were run. The plates were stained with 0.05% Rhodamine 6G in 95% ethanol and visualized under uv light, and appropriate zones scraped into scintillation vials. Cab-O-Sil (Packard Instruments) was used as a gelling agent to keep the silica gel suspended for scintillation counting (Snyder and Stephens, 1962).

Phospholipase A_2 Treatment of Lipid Extract. Lipid extracts were treated with *Crotalus adamanteus* venom (Sigma Chemical Co.) as a source of phospholipase A_2. After dissolving the extract in 2 ml of diethyl ether, 50 μl of a 1 mg/ml buffered solution of venom (0.22 M NaCl, 20 mM $CaCl_2$, and 1 mM EDTA, pH 7.5) (Wells and Hanahan, 1969) was added to the extract. This mixture was purged with nitrogen, sealed, and incubated for 60 min at room temperature with shaking. Reactions were stopped by extraction with chloroform–methanol (2:1, v/v). The amount of radioactively labeled free

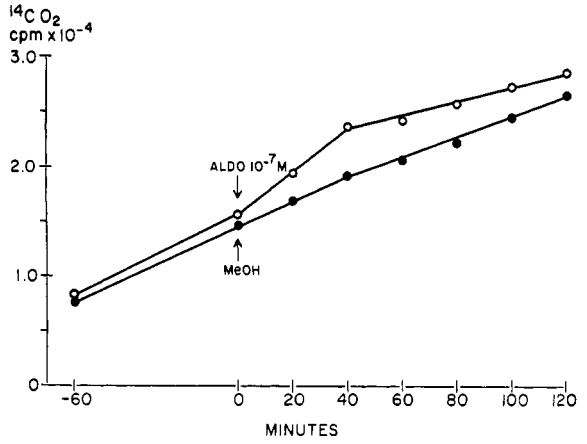

FIGURE 1: Time course of the decarboxylation of [1-^{14}C]glucose. Toad bladders were prepared as described in Materials and Methods. Aliquots of the incubation medium were removed at appropriate times and the [^{14}C]CO_2 content was determined. Aldosterone (○) in methanol or carrier methanol (●) was added to matched-paired tissue at the time indicated. Data are plotted as the cumulative [^{14}C]CO_2 produced during the incubation.

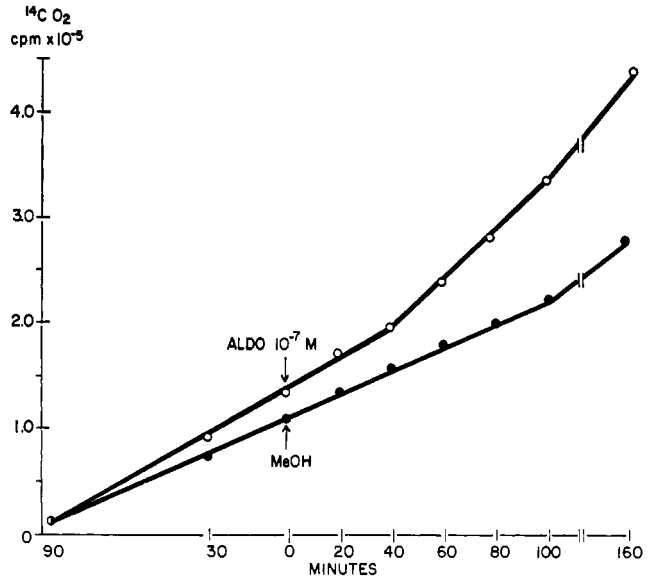

FIGURE 2: Time course of the decarboxylation of [6-^{14}C]glucose. Experiments were performed as described for Figure 1.

fatty acid released was determined by analysis of the reaction products using the Freeman and West tlc system. Aliquots of the labeled lipid extract were also analyzed directly without treatment with snake venom to determine the amount of [^{14}C]free fatty acid present. This correction was applied in the final calculation of free fatty acid release due to phospholipase action. Under these conditions, the phospholipase A reaction was complete by 50 min.

Gas–Liquid Chromatography of the Methyl Esters of Phospholipids. Lipids were extracted from matched hemibladders of eight toads treated for 30 min, 90 min, or 6 hr with aldosterone or carrier methanol. Phospholipids were isolated as described above and the methyl esters 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. The mass response was quantitated with the aid of an Infotronics digital integrator Model CRS-10AB. Qualitative tentative identification of the various peaks was made on the basis of (1) plots of log retention time *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 newly synthesized fatty acids, [^{14}C]fatty acid methyl esters derived from the phospholipids of tissue incubated in the presence of [2-^{14}C]pyruvate were collected utilizing a Varian Model 96-0000-18 10:1 glass splitter–collector.

Alteration of the Latent Period by the Action of Snake Venom on Intact Bladders. Hemibladders, depleted of endogenous aldosterone as described above, were mounted in an Ussing-type voltage clamp (Sharp and Leaf, 1964) in which each hemibladder is clamped in a double chamber allowing independent measurement of short-circuit current from two halves of each hemibladder. The chambers were filled with Ling–Ringer phosphate containing 4 mM glucose, and short circuit was recorded continuously. Either the serosal or mucosal bathing solution of one side of the chamber received snake venom in a final concentration of 150 μg/ml. After about 30–40 min when the short-circuit current had restabilized, 10^{-7} M aldosterone was added to both sides of the chamber. The experiment was allowed to continue for at least 18 hr. The duration of the latent period was then determined on the control and the venom-treated sides.

Results

Previous studies of glucose decarboxylation in the toad bladder had shown that after a lag time of about 1 hr, aldosterone stimulated [^{14}C]CO_2 from [6-^{14}C]glucose (Kirchberger *et al.*, 1968). However, because we felt that lipid metabolism might be involved in the action of this hormone and because the hexose monophosphate shunt is regarded as a source of reducing equivalents for lipogenesis, we undertook a reinvestigation of these parameters of substrate utilization. Using an alternate technique, we have found that within 20 min after the addition of aldosterone, increased [^{14}C]CO_2 evolution from [1-^{14}C]glucose occurs. In three consecutive experiments the rate of [^{14}C]glucose decarboxylation increased 52.0 ± 16.3% during the first 40 min after hormone addition. This increase is sustained for an additional 20–40 min after which, as had been previously observed (Kirchberger *et al.*, 1968), the rate of decarboxylation of this isotope decreased to less than the rate before hormone addition. The results of a typical experiment are plotted in Figure 1. During the time interval when [^{14}C]CO_2 evolution from [1-^{14}C]glucose is increasing, there is no change in the decarboxylation of [6-^{14}C]-glucose (Figure 2). However, after a period of 40–60 min, an increase in [^{14}C]CO_2 production from [6-^{14}C]glucose is observed concomitant with an inhibition of [^{14}C]CO_2 evolution from [1-^{14}C]glucose. The stimulation of [^{14}C]CO_2 evolution from [1-^{14}C]glucose during the first hour after addition of hormone, at a time when there is no change in [^{14}C]CO_2 production from [6-^{14}C]glucose, we interpreted as a stimulation of the hexose monophosphate shunt.

Lipid metabolism was then investigated during the period of increased [^{14}C]CO_2 evolution from [1-^{14}C]glucose. Within 20 min after its addition, aldosterone induced striking changes

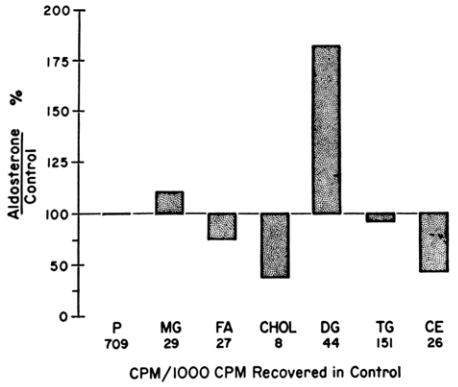

FIGURE 3: Pattern of lipid labeling after a 20-min exposure to aldosterone. Scraped epithelial cells were preincubated in uniformly labeled [^{14}C]glucose for 60 min. Aldosterone (10^{-7} M) in methanol or carrier methanol was then added to the appropriate flasks and the incubation allowed to continue. Lipids were then extracted and separated into the principal lipid classes on silica gel G as described in Materials and Methods. Appropriate zones of the thin-layer chromatography plate were scraped into scintillation vials and the ^{14}C content was determined. The ordinate is the counts per minute in each lipid class per 1000 cpm recovered in aldosterone-treated epithelial cells divided by the counts per minute in each lipid class per 1000 cpm recovered in paired control cells. The data are expressed as a percentage. P, phospholipids; M, monoglycerides, FA, fatty acids; CHOL, cholesterol; DG, diglycerides, TG, triglycerides; CE, cholesterol esters.

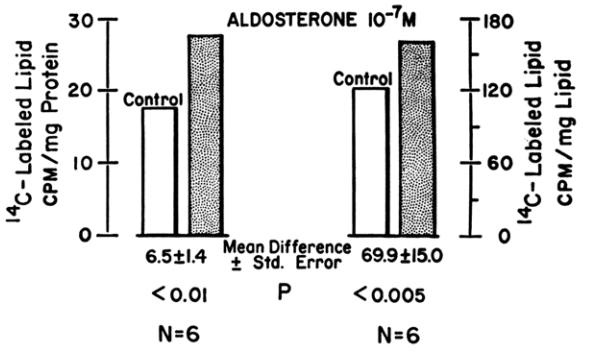

FIGURE 5: The effect of aldosterone on [2-^{14}C]pyruvate incorporation into lipid. Matched hemibladders were preincubated for 60 min in [2-^{14}C]pyruvate before addition of aldosterone (10^{-7} M) in methanol or carrier methanol. The incubation was allowed to continue for 60 min before extraction of lipids.

in the pattern of lipid labeling. With 4 mM glucose as substrate and [U-^{14}C]glucose as label, addition of aldosterone to scraped epithelial cells consistently led to an accumulation of radioactivity in the diglyceride fraction of a total lipid extract (Figure 3). If this same incubation was carried out for 90 min, the hormone induced an accumulation of radioactivity in the phospholipid fraction with a concomitant decrease in the extent of labeling of the other principal lipid classes (Figure 4). These data, taken together with the stimulation of [^{14}C]CO_2 evolution from [1-^{14}C]glucose, indicated that the steroid was inducing the synthesis of phospholipid.

Utilization of a scraped cell preparation allowed localization of the hormonal effect to the epithelial cell layer. However, precise quantitative assessment of the degree of lipid synthesis was not possible because of the clumping of the cell preparation and the consequent difficulty in measuring equal aliquots of cells. Further studies were, therefore, performed utilizing whole intact bladders. To localize the site of incorporation of radioactive label to the fatty acid moiety of complex lipids, [2-^{14}C]pyruvate was used as radioactive precursor since under these experimental conditions greater than 90% of the radioactivity in ^{14}C-labeled lipid was present as phospholipid and could be recovered as fatty acid methyl esters after saponification. As shown in Figure 5, hormone treatment stimulated pyruvate incorporation into lipid when either total protein or total lipid in the sample was used as a basis for comparison. It should be noted, however, that in these experiments the lipid to protein ratio remained constant (control 0.1308 ± 0.014 *vs.* aldosterone treated 0.1438 ± 0.024) indicating that the hormonally induced lipid constituted a small portion of the total tissue lipid.

To characterize the lipid synthesized after hormone addition, the pattern of phospholipid labeling was investigated. As shown in Table I, aldosterone treatment did not cause the preferential labeling of any specific phospholipid. Thus, it appears that the fatty acids induced by hormone treatment are

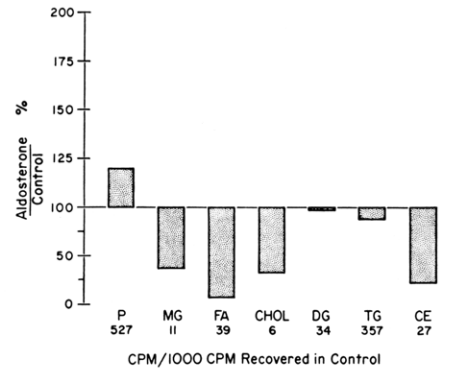

FIGURE 4: Pattern of lipid labeling after a 90-min exposure to aldosterone. The experiment was performed as described in Figure 3, except that the incubation was carried out for 90 min.

TABLE I: Pattern of Phospholipid Labeling in Aldosterone-Treated and Control Hemibladders.

Lipid	% of Total [^{14}C]Lipid Labeled[b]	
	Aldosterone	Control
LPC[a]	0.7 ± 0.7	0.7 ± 0.6
SPH	1.4 ± 1.1	1.6 ± 0.8
PC	8.0 ± 6.7	5.9 ± 4.9
PI	4.1 ± 3.6	4.5 ± 5.4
PS	4.6 ± 5.2	5.3 ± 4.4
PE	78.3 ± 7.0	78.5 ± 6.5
SF	2.8 ± 1.1	2.6 ± 1.5

[a] Abbreviations used are: LPC, lysophosphatidylcholine; SPH, sphingomyelin; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; SF, solvent front. [b] Data presented are the mean plus and minus standard deviation of five separate experiments. Bladders were preincubated for 60 min in [2-^{14}C]pyruvate before addition of aldosterone (10^{-6} M) in methanol or carrier methanol. The incubation was continued for 60 min; lipids were then extracted and fractionated into individual phospholipid classes.

TABLE II: Relative Specific Activities of [2-^{14}C]Pyruvate-Labeled Fatty Acids Derived from Toad Bladder Phospholipids after Aldosterone Treatment.[a]

Fatty Acid	30 min	90 min	6 hr
16	1.8	0.5	1.6
16:1,2	7.9	86.4	2.3
18	4.9	5.1	3.2
18:1	7.0	2.6	22.2
18:2	6.3	1.7	2.8
20	1.2	1.1	2.9
18:3	1.1	0.8	2.5
20:2	0.4	0.6	6.5
20:4, 22:1	1.1	0.8	4.0
20:5	0.2	0.3	0.7
24:1, 22:5ω6	1.7	1.2	4.7
22:5ω3, 22:6	1.0	1.4	3.5

[a] Data are represented as the ratio of the specific activities of fatty acids in aldosterone-treated tissue as compared to control tissue. Bladders were preincubated for 60 min in [2-^{14}C]pyruvate before addition of aldosterone (10^{-7} M) in methanol or carrier methanol. The incubation was continued for 30 min, 90 min, or 6 hr, respectively, before extraction and preparation of fatty acid methyl esters. The specific activity of individual fatty acids was calculated by dividing the mass response of the detector by the amount of radioactivity recovered.

TABLE III: Fatty Acid Composition of Toad Bladder Phospholipids Derived from Aldosterone-Treated and Control Tissue.[a]

Fatty Acid	Weight % Fatty Acid in Aldosterone	Weight % Fatty Acid in Control
14	2.45 ± 0.76	2.28 ± 0.57
14:1	5.07 ± 0.34	5.00 ± 0.26
16	15.86 ± 1.84	15.65 ± 1.13
16:1, 2	6.09 ± 0.46	6.04 ± 0.58
18	10.02 ± 0.03	10.28 ± 0.49
18:1	13.62 ± 1.23	13.78 ± 1.73
18:2	16.36 ± 0.59	16.68 ± 0.59
20	2.68 ± 1.06	2.58 ± 0.37
18:3	1.58 ± 0.15	1.49 ± 0.03
20:2	2.52 ± 0.33	2.33 ± 0.21
20:4, 22:1	14.73 ± 1.45	15.15 ± 0.67
20:5	2.25 ± 0.50	1.82 ± 0.29
24:1	3.87 ± 0.43	3.03 ± 0.33[b]
22.5ω6	0.96 ± 0.14	0.45 ± 0.15[c]
22:5ω3	1.93 ± 0.56	0.76 ± 0.18[c]
22:6	0.87 ± 0.10	0.37 ± 0.13[b]

[a] Values are the mean plus and minus standard deviation of three separate determinations. [b] $P < 0.05$. [c] $P < 0.02$. Matched hemibladders were exposed to aldosterone (10^{-7} M) for 6 hr before extraction.

not incorporated preferentially into any specific phospholipid class. The possibility that newly synthesized fatty acid was being esterified preferentially in a specific position of phospholipid molecules was explored by utilizing phospholipase A_2. When [2-^{14}C]pyruvate-labeled lipid extracts were treated with this enzyme, in three separate experiments lipids from hormone treated tissue released on the average 3.7 times as much radioactivity into free fatty acids as did untreated control tissue extracts (aldosterone treated 119 ± 55 cpm released per 1000 cpm in lipid *vs.* control 32 ± 21 cpm released per 1000 cpm in lipid).

When gas–liquid chromatography is carried out on fatty acid methyl esters derived from toad bladder phospholipids, no change in the weight percentage of any fatty acid class is observed within the first 90 min after hormone addition. However, the specific activity of fatty acid methyl esters with chain length up to 18:2 is increased by aldosterone treatment within 30 min. This increase in specific activity is greatest in the 16:1,2-methyl esters; 30 min after aldosterone addition, there is a 7.9-fold and by 90 min an 86.4-fold increase in the specific activity of these methyl esters (Table II). Although 6-hr exposure to aldosterone does not alter the weight percentage of fatty acids with chain length up to 18 carbons, the weight percentage of several long-chain polyunsaturated fatty acids is increased in hormone-treated tissue phospholipids (Table III). The specific activity of these polyunsaturated fatty acids is also increased 3.5- to 4.7-fold by aldosterone treatment (Table II). Thus after 6 hr, aldosterone causes an alteration in the fatty acid composition of toad bladder membrane phospholipids.

One possible mechanism by which aldosterone might be altering the composition of membrane phospholipids is through activation of a phospholipid → lysophospholipid → phospholipid cycle mediated by the enzymes phospholipase A and lysophospholipid–acylase, respectively. If this were the case, treatment of the intact bladder with phospholipase A, before addition of aldosterone, might be expected to shorten the latent period that occurs before the usually observed hormone-stimulated increase in Na^+ transport. When this experiment was performed, treatment of the serosal surface of the tissue with a phospholipase A containing solution of snake venom significantly reduced the latent period in five consecutive experiments. On the control side, the latent period averaged 93 min; with phospholipase A treatment, the latent period was reduced 21.4 ± 5.5 min ($P < 0.02$). Addition of phospholipase A to the mucosal surface of the tissue did not alter the latent period significantly in parallel experiments ($P > 0.8$). When tissue was prelabeled for 60 min with [^{32}P]PO_4 and subsequently incubated in fresh buffer containing 150 μg/mole of snake venom for 45 min, the ^{32}P content of lysophosphatidylcholine increased from 2.1 ± 0.7% of the total [^{32}P]phospholipid in control tissue to 15.9 ± 6.3% in venom treated tissue. Thus under our experimental conditions increased lysophospholipid content results from snake venom treatment.

Discussion

The present report provides the first evidence for an effect of aldosterone on toad bladder membrane phospholipid metabolism. Within the first hour after its addition, aldosterone stimulates the conversion of [2-^{14}C]pyruvate into fatty acids. These labeled fatty acids are not preferentially incorporated into any specific phospholipid but are esterified preferentially in the 2 position of phospholipids. Although no

change in the weight percentage of any phospholipid fatty acid class is observed within the first 90 min after aldosterone addition, the specific activity of fatty acids of chain length up to 18:2 is increased by aldosterone treatment within 30 min. After 6 hr, aldosterone induces an increase in both the weight percentage and specific activity of several long-chain polyunsaturated fatty acids.

In previous reports, the decarboxylation of [1-^{14}C]glucose has been shown to be inhibited after at least 1 hr of hormone treatment (Kirchberger *et al.*, 1968; Handler *et al.*, 1969b). We have confirmed this observation in the present study. However, to date, no explanation for this finding has been advanced. This inhibition of [1-^{14}C]glucose decarboxylation could result from an activation of the Embden–Myerhoff pathway and the Krebs cycle secondary to increased energy requirements for active sodium transport. A hormone-induced shift in the cytoplasmic NAD:NADH ratio to a more reduced state after 6 hr of aldosterone treatment has been reported (Handler *et al.*, 1969b). This altered redox state should be reflected in the cytoplasmic NADP–NADPH couple as well (Krebs and Veech, 1968). Thus, before the shift in cytoplasmic redox state, increased decarboxylation of [1-^{14}C]glucose is seen and reflects increased hexose monophosphate shunt activity to supply reducing equivalents for fatty acid synthesis. After activation of glycolysis and the Krebs cycle, the altered redox state in the cytoplasm serves to inhibit flux through the hexose monophosphate shunt, reducing equivalents for lipid synthesis then being supplied *via* a transhydrogenase shuttle involving a cytoplasmic NAD-linked malate dehydrogenase and malic enzyme.

The increased labeling of the diglyceride fraction observed within 20 min after addition of aldosterone provided an initial clue that the hormone was altering lipid metabolism. An exact interpretation of this finding is not, however, possible from these studies. The observed accumulation of radioactivity in this fraction could be due to increased *de novo* synthesis of diglycerides which eventually are converted to phospholipids, or from an inhibition of the conversion of diglycerides to phospholipids.

Fatty acyl-CoA labeled during the first hour after hormone addition might be acylating lysophospholipids directly resulting in the labeling of phospholipids. This possibility is supported by both the striking increase in the specific activity of phospholipid fatty acids of 16 and 18 carbons induced by aldosterone treatment for only 30 min and the evidence that pretreatment of the serosal surface of the toad bladder with phospholipase A resulted in a significant shortening of the time interval from addition of aldosterone until the increase in sodium transport and increased lysophospholipid formation.

Our present evidence is inadequate to decide whether the hormone stimulates complete *de novo* synthesis of phospholipids or the hydrolysis of phospholipids to lysophospholipids followed by their reesterification to phospholipid with a new fatty acid in the 2 position. The requisite enzymes for the lysophospholipid–phospholipid cycle have been identified in toad bladder (Rosenbloom and Elsbach, 1968a,b). The present data represent the first possible evidence for the hormonal control of this cycle. The exact relationship between the stimulation of the synthesis of a small pool of 16- and 18-carbon fatty acids and increased fatty acid incorporation into the 2 position of phospholipids during the first 60–90 min after hormone addition and the increase in both the weight percentage and specific activity of several classes of long-chain polyunsaturated phospholipid fatty acids after 6 hr of hormone treatment remains to be determined. This hormone-induced alteration in the fatty acid metabolism of toad bladder membrane phospholipids, however, raises two questions. (1) Can this alteration of membrane fatty acid metabolism explain the biological effects of the hormone? (2) How are these changes observed 6 hr after hormone addition, as well as the effects on phospholipid metabolism observed as early as 20–30 min after aldosterone addition, related to previous results indicating that aldosterone exerts its effects by altering RNA and protein synthesis?

With regard to the second question, the evidence in support of the hypothesis that aldosterone acts primarily by regulating gene expression is based primarily on experiments with inhibitors of RNA and protein synthesis. Pretreatment of the tissue with either class of inhibitors leads to an inhibition of hormone action (Edelman *et al.*, 1963; Edelman, 1968). However, it has become increasingly clear that these inhibitors may not be completely specific in their action. There is also increasing evidence that control of gene expression in eukaryotic cells is considerably more complex than in bacterial cells (Schimke, 1969). Many key enzymes turn over with a short half-life. If the activity of an enzyme with a relatively short half-life were essential for the response to hormone, then treatment of the tissue with inhibitors of RNA or protein synthesis would lead to an inhibition of the response to hormone, even though the hormone had not altered the rate of protein synthesis. Thus, an equally valid conclusion to be drawn from the studies with these inhibitors in the toad bladder is that an enzyme with a short half-life is involved in the hormone-sensitive process. This means that net synthesis of new proteins is not a necessary part of the hormonally induced changes in the toad bladder.

The present findings concerning the effect of aldosterone upon lipid metabolism and particularly the increase in specific classes of the fatty acids in phospholipids could account for a number of the effects of the hormone on this tissue. Changes in membrane fatty acid composition have been observed in a variety of tissues from goldfish acclimatized at different temperatures (Knipprath and Mead, 1968; Kemp and Smith, 1970), and these differences in fatty acid composition are associated with differences in the activity of the membrane-bound ATPase (Smith and Kemp, 1969). A similar situation may exist in the toad bladder and account for the changes in Na^+ transport induced by aldosterone. It has also been found in artificial phospholipid membranes that an increase in the content of unsaturated fatty acids leads to changes in permeability (de Gier *et al.*, 1968). Similar changes in this biological membrane may account for the increased permeability of aldosterone-treated bladders in the presence of vasopressin (Handler *et al.*, 1969a). A change in the lipid environment of the Na^+–K^+-activated ATPase could also account for the increased susceptibility of aldosterone-treated tissue to inhibition by ouabain (Goodman *et al.*, 1969). The increased requirement for reducing equivalents caused by the aldosterone-induced increase in fatty acid synthesis as well as an increasing degree of unsaturation of membrane fatty acids could also account for the increased sensitivity of aldosterone-treated bladders to the toxic effects of hyperbaric oxygen. Although aldosterone produces only about a 3% change in the weight percentage of phospholipid fatty acids in whole toad bladders, the evidence from this study and from all other studies of aldosterone action in the toad urinary bladder indicates that the epithelial cell layer, which comprises at most 10% of the mass of the toad bladder (Peachey and Rasmussen, 1961), is the site of aldosterone action. Thus, it is not

surprising that the aldosterone-induced changes in whole bladder phospholipid fatty acid composition are small. If the observed changes were to occur specifically in the serosal plasma membrane of the epithelial cells, they could easily account for the observed effects of aldosterone on toad bladder function.

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Studies on the Soluble 17β-Hydroxysteroid Dehydrogenase from Human Placenta. Evidence for a Subunit Structure*

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ABSTRACT: The molecular weight of the 17β-hydroxysteroid dehydrogenase, estimated from polyacrylamide electrophoresis in 0.1% sodium dodecyl sulfate, is 35,000. This is approximately one-half the value obtained in an earlier study of the active enzyme and suggests that the enzyme is composed of subunits. Cross-linking with dimethyl suberimidate followed by electrophoresis in sodium dodecyl sulfate gives, in addition to a band of molecular weight 37,000, a second protein band with a molecular weight of approximately 73,000. The molecular weight calculated using the Stokes radius and the sedimentation coefficient of the active enzyme is 72,000. The elution volume of the 17β-hydroxysteroid dehydrogenase on Sephadex G-200 filtration is markedly affected by the conditions of filtration: acidic pH or low temperature (4°) produces aggregation of the enzyme while sodium dodecyl sulfate leads to dissociation.

Early attempts to purify the 17β-hydroxysteroid dehydrogenase from human placenta were hindered by its instability (Langer and Engel, 1958; Talalay *et al.*, 1958), but addition of glycerol to solutions of the enzyme protected it from inactivation and permitted an extensive purification (Jarabak *et al.*, 1962). Further studies revealed that the instability of the enzyme was due to a partially reversible cold inactivation, during which the enzyme underwent aggregation (Jarabak *et al.*, 1966). Taking precautions to protect the enzyme from cold inactivation, three groups of investigators (Descomps *et al.*, 1968; Jarabak, 1969; Karavolas *et al.*, 1970)

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