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Effects of an Acetyl-Coenzyme A Carboxylase Inhibitor and a Sodium-Sparing Diuretic on Aldosterone-Stimulated Sodium Transport, Lipid Synthesis, and Phospholipid Fatty Acid Composition in the Toad Urinary Bladder†

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ABSTRACT: A correlative study of the effects of two agents, 2-methyl-2-[*p*-(1,2,3,4-tetrahydro-1-naphthyl)phenoxy]propionic acid (TPIA) and amiloride, on aldosterone-induced alterations in Na^+ transport, lipid synthesis, and phospholipid fatty acid composition has been carried out in the toad urinary bladder. TPIA, an inhibitor of acetyl-CoA carboxylase, inhibits aldosterone-stimulated Na^+ transport as well as hormone-induced lipid synthesis and the increase in weight percentage of phospholipid long-chain polyunsaturated fatty acids. Amiloride, a diuretic which blocks sodium entry into the transporting epithelium, does not alter aldosterone's effects on lipid and fatty acid metabolism but prevents the hormone-induced increase in Na^+ transport. These results support the conclusion that aldosterone increases Na^+ transport in the toad urinary bladder by altering membrane fatty acid metabolism and that the lipid biosynthetic events following aldosterone treatment are a primary response to the hormone and not secondary to increased Na^+ transport.

Transepithelial Na^+ transport across the isolated toad urinary bladder is stimulated by two hormones: the peptide, vasopressin (Leaf et al., 1958), and the steroid, aldosterone (Crabbé, 1961). The two act synergistically, but their time courses of action are quite different. Vasopressin increases Na^+ transport, as well as water permeability, after a delay of only 2–3 min with the maximal response being reached within 15 min; aldosterone stimulates only Na^+ transport after a latent period of 45–90 min with the peak response being observed in 4–6 hr.

A model to account for aldosterone action has been developed in which the steroid activates one or more specific genes in bladder epithelial cell nuclei leading to an increase in the synthesis of one or more specific proteins involved in transcellular Na^+ transport (Edelman et al., 1963). In keeping with this model, inhibitors of either mRNA or protein synthesis block the aldosterone-induced rise in Na^+ transport without blocking the vasopressin-induced increase (Edelman et al., 1963; Chu and Edelman, 1972).

Our own studies of aldosterone action began with the observation that pretreatment of the toad bladder with aldosterone led to a dramatic increase in its sensitivity to hyperbaric oxygen (Allen et al., 1973). In aldosterone-treated tissue an increase in the partial pressure of O_2 led to a reversible inhibition of Na^+ transport without an inhibition of cell metabolism or fall in ATP content. These data led to an investigation of the possible effects of aldosterone upon membrane lipid turnover. In previous work (Goodman et al., 1971) we observed an aldosterone-induced increase in incorporation of precursor molecules (pyruvate or glucose) into membrane fatty acids before any detectable change in transcellular Na^+ transport was observable. Furthermore, the weight percentage of phospholipid long-chain polyunsaturated fatty acids increased after 6 hr of aldosterone treatment, concomitant with the maximal increase in Na^+ transport. We have recently found that following aldosterone addition a stimulation of endogenous phospholipase activity and a specific increase in oleic acid metabolism occur within 30 min and persist for as long as 6 hr (Goodman et al., 1975).

These results could mean (1) aldosterone has a primary effect upon the turnover of membrane fatty acids and this is the basis of its effects upon transcellular Na^+ transport; (2) aldosterone alters the synthesis of one or more membrane transport proteins which, in order to be incorporated into

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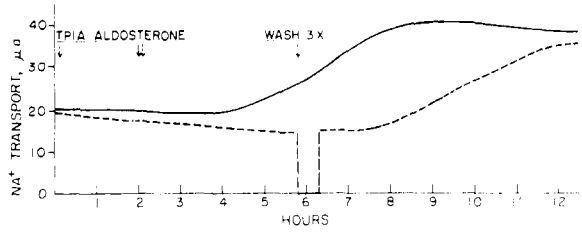

FIGURE 1: Effect of TPIA and aldosterone on sodium transport in toad bladder as measured by short-circuit current. TPIA (2 m*M*) was added to the mucosal side of one of the chambers, and 2 hr later aldosterone (10^{-7} *M*) was added to both serosal and mucosal sides of both chambers. Four hours after aldosterone addition both mucosal and serosal sides of the TPIA-treated tissue were washed three times and aldosterone was re-added.

the membrane, require a new membrane lipid environment; or (3) the increase in phospholipid long-chain polyunsaturated fatty acids is secondary to the aldosterone-induced increase in Na^+ transport.

To gain further insight into the role of altered membrane lipid metabolism in aldosterone action and possibly help choose among these three alternatives, studies have been carried out with two inhibitors: 2-methyl-2-[*p*-(1,2,3,4-tetrahydro-1-naphthyl)phenoxy]propionic acid (TPIA),[1] an inhibitor of fatty acid biosynthesis (Maragoudakis, 1969), and amiloride, a specific inhibitor of mucosal Na^+ entry in the intact bladder (Bentley, 1968).

Mucosal addition of TPIA inhibited the aldosterone-induced increase in Na^+ transport without drastically altering basal Na^+ transport or the vasopressin-induced increase in either Na^+ transport or water permeability. TPIA also blocked both fatty acid chain elongation in control tissue and the aldosterone-induced change in the content of phospholipid long-chain polyunsaturated fatty acids without altering either RNA or protein synthesis. In contrast, mucosal addition of amiloride reduced basal Na^+ transport, blocked both AVP and aldosterone-induced increases in Na^+ transport, but did not block the aldosterone-induced changes in phospholipid fatty acid composition.

Materials and Methods

Sodium Transport. Urinary bladders from female toads (*Bufo marinus*, National Reagents, Bridgeport, Conn.) were prepared and mounted as previously described (Goodman et al., 1969). Active transepithelial sodium transport was measured as short-circuit current (Ussing and Zerahn, 1951).

RNA and Protein Synthesis. RNA and protein synthesis were analyzed in double-label experiments using [U-^{14}C]leucine and [^{3}H]uridine (Amersham/Searle, Arlington Heights, Ill.), 0.01 m*M*; specific activity, 30 μCi/mmol. These incubations, and all other incubations involving TPIA or amiloride, were carried out with hemibladders mounted as bags (Bentley, 1958). The mucosal bathing solution (2 ml) was prepared by dissolving TPIA (a gift of Ciba Geigy, Summit, N.J.) in dimethyl sulfoxide and adding this concentrate to Ling-Ringer phosphate buffer (Ling, 1962) to a final concentration of 2 m*M* TPIA and 0.3% v/v Me_2SO. After a 2-hr preincubation with TPIA and 0.01 m*M* carrier leucine and uridine, fresh serosal bathing solution was added. Aldosterone (Calbiochem, La-Jolla, Calif.), 10^{-7} *M*, or methanol was distributed in a manner to generate four types of incubations: control, aldosterone, TPIA, and TPIA + aldosterone. After an initial 30-min exposure to aldosterone and serosal buffer was replaced with buffer containing the radioactive substrates. After an additional 30 min the tissue was frozen in liquid nitrogen and pulverized with a stainless-steel mortar and pestle kept in Dry Ice. Protein and RNA were extracted from the bladders by a modification of the procedure of Hutchinson and Porter (1970). Each powdered bladder was placed in a 12-ml centrifuge tube, 2 ml of cold 0.3 *N* perchloric acid was added, and the extract was then mixed thoroughly. The tubes were centrifuged for 6 min at 1250*g*, the sediment was washed two times with 2 ml of cold 0.3 *N* perchloric acid, and the supernatants were discarded. The precipitate was washed one time with 2 ml of cold 80% ethanol to remove excess perchloric acid. Extracts were dissolved in sodium hydroxide (1.0 *N*) and aliquots were taken for double-label analysis (Kobayashi and Maudsley, 1970) and protein determination (Lowry et al., 1951).

Hydroosmotic Response. Osmotic water permeability of the tissue was determined gravimetrically (Bentley, 1958). Bags were prepared and the rate of water movement from the mucosal surface (containing a 1:5 strength Ling-Ringer buffer) to the serosal surface (containing full strength buffer) was determined by weighing the hemibladders every 10 min for the following periods: (1) 1-hr base line before the addition of TPIA; (2) 2-hr period of exposure to 2 m*M* TPIA added mucosally to one-half of the bladders; and (3) a 40-min exposure to 65 mU/ml of vasopressin (Pitressin, Parke Davis, Detroit, Mich.) added to the serosal bath.

Lipid Studies. Tissue was initially exposed to TPIA (2 m*M*, mucosal) or amiloride (Merck Sharp and Dohme, West Point, Pa.), 0.05 m*M*, mucosa, for 2 hr in the presence of 0.05 m*M* Na^+ acetate. The serosal bath was then removed and fresh buffer, containing [U-^{14}C]acetate (0.1 mCi/mmol, Amersham/Searle, Arlington Heights, Ill.), was added to all bladders; aldosterone, 10^{-7} *M*, was added to half. After 30 min the bladders were frozen and powdered as described above. The incorporation of [^{14}C]acetate into lipid and the specific activities of phospholipid fatty acids were determined on tissue lipid extracts as previously described (Goodman et al., 1971).

Results

Effects of TPIA on Basal and Stimulated Na^+ Transport and Bulk H_2O Flow. Addition of 2 m*M* TPIA to the mucosal surface of the tissue had little effect upon the basal rate of Na^+ transport; however, addition of as little as 0.5 m*M* TPIA to the serosal surface led to an immediate fall in Na^+ transport. It was not possible to find a concentration of TPIA which , when added to the serosal bath, did not alter basal transport but did block aldosterone action. Hence, all the studies with this inhibitor were carried out utilizing 2 m*M* TPIA added only to the mucosal bathing medium.

A 2-hr pretreatment with TPIA did not alter the change in Na^+ transport seen after vasopressin but did inhibit the rise in short-circuit current normally seen after aldosterone addition (Figure 1). The hydroosmotic response to vasopressin was not altered by pretreatment of this tissue with either TPIA (Table I) or amiloride (Bentley, 1968).

Effects of TPIA on Protein and RNA Synthesis. Pretreatment of bladders for 2 hr with mucosal TPIA did not alter the rate of incorporation of either [^{14}C]leucine into protein or [^{3}H]uridine into RNA in the presence or absence of aldosterone (Table II).

[1] Abbreviation used is: TPIA, 2-methyl-2-[*p*-(1,2,3,4-tetrahydro-1-naphthyl)phenoxyl]propionic acid.

Table I: The Hydroosmotic Response of the Toad Urinary Bladder to Vasopressin in the Presence of TPIA.[a]

	Period		
	I	II	III
Control[b]	0.37 ± 0.09	0.41 ± 0.09	35.16 ± 3.56
TPIA	0.42 ± 0.05	0.27 ± 0.19	36.12 ± 4.48

[a] Sacs containing a 1:5 dilution of buffer on the mucosa and full strength buffer on the serosa were weighed every 10 min for the following periods: (I) a 60-min base line; (II) 120-min incubation with and without 2 m*M* TPIA added to the mucosa; and (III) a 40-min exposure to 65 mU/ml of vasopressin added to the serosa. [b] Weight loss in milligrams/minute. Each group consisted of eight hemibladders. Data are the mean ± standard deviation of each group.

Table II: Radioactive Precursor Incorporation into Protein and RNA.[a]

	[^{14}C] Leucine × 10^{-2}	[^{3}H] Uridine × 10^{-4}
Control	2.65 ± 0.46	1.47 ± 0.14
TPIA	2.57 ± 0.42	1.53 ± 0.17
Aldosterone	2.21 ± 0.43	1.16 ± 0.20
Aldosterone + TPIA	2.09 ± 0.14	1.28 ± 0.06

[a] Tissue was incubated 2 hr with or without 2 m*M* TPIA (mucosa). Aldosterone (10^{-7} *M*) was then added to half the control tissue and half the TPIA-treated tissue for 30 min (matched pairs: control and TPIA, aldosterone and aldosterone plus TPIA). [U-^{14}C] Leucine and [^{3}H] uridine were added to all samples for an additional 30-min incubation. Values are the means plus and minus the standard deviation of six determinations (disintegrations per minute per milligram of protein). No significant differences were observed when tissue was incubated with and without TPIA.

Table III: The Effect of TPIA and Amiloride on [U-^{14}C] Acetate Incorporation into Lipid in the Presence and Absence of Aldosterone.[a]

	(*n*)	Mean	Mean Difference ± SE of Paired Sample	*P*	Ratio
TPIA	(13)	311.2	131.9 ± 43.3	0.005	0.70
Control		443.2			
Aldosterone	(13)	344.1	61.5 ± 23.2	0.01	1.21
Control		282.4			
TPIA + aldosterone	(13)	329.8	6.7 ± 21.1	ND	1.02
TPIA		323.1			
Amiloride	(7)	504.0	82.3 ± 41.1	0.05	1.19
Control		422.6			
Aldosterone	(7)	525.2	78.4 ± 36.6	0.05	1.17
Control		445.3			
Amiloride + aldosterone	(7)	494.6	51.3 ± 15.1	0.01	1.12
Amiloride		443.3			

[a] Matched hemibladders were incubated with or without 2 m*M* TPIA (mucosa) or with or without 0.05 m*M* amiloride (mucosa) for 2 hr. [U-^{14}C] Acetate was then added to all tissues and aldosterone (10^{-7} *M*) added to appropriate tissue for an additional incubation of 30 min before extraction. Data are expressed as the mean incorporation (counts per minute per milligram of protein).

Effects of TPIA on Basal and Aldosterone-Stimulated Lipid Metabolism. When the bladders were preincubated for 2 hr with 2 m*M* mucosal TPIA and then [^{14}C]acetate was added for 0.5 hr, the rate of incorporation of [^{14}C]acetate into total lipid was approximately 70% of that seen in the absence of TPIA (Table III). When the methyl esters of the labeled phospholipid fatty acids were separated by gas

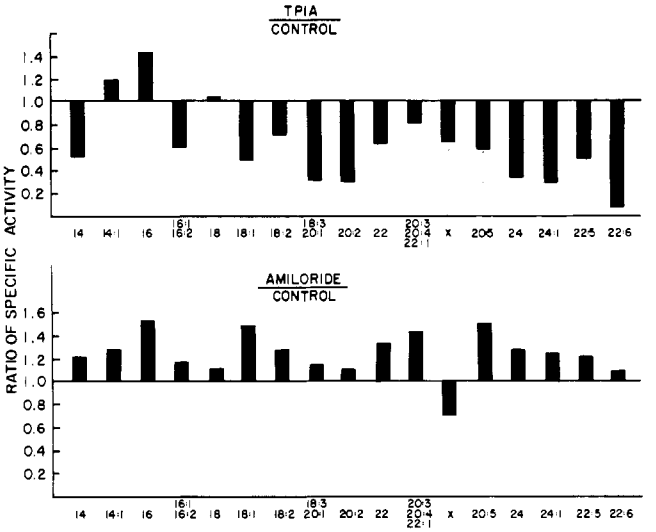

FIGURE 2: Relative specific activities of [U-^{14}C]acetate-labeled fatty acids derived from toad bladder phospholipids. Eight hemibladders were treated for 2 hr with either TPIA (2 m*M*, mucosa) or amiloride (0.05 m*M*, mucosa); matched pairs served as controls. [U-^{14}C]Acetate was added 30 min before extraction and preparation of fatty acid methyl esters. The specific activity of the fatty acids was calculated by dividing the amount of radioactivity recovered by the mass response of the detector. The data presented are derived from two separate incubations, each run in triplicate on the gas–liquid chromatograph.

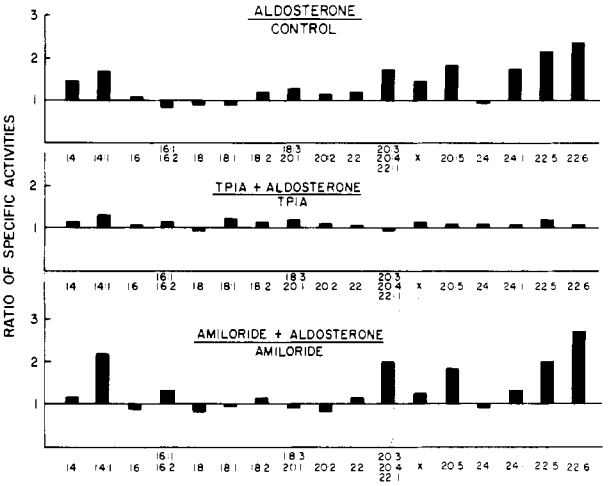

FIGURE 3: Relative specific activities of [U-^{14}C]acetate-labeled fatty acids derived from toad bladder phospholipids. Untreated, TPIA- (2 m*M*, mucosa), and amiloride- (0.05 m*M*, mucosa) treated hemibladders were incubated for 2 hr before addition of aldosterone to one hemibladder of each pair and [U-^{14}C]acetate to all tissue for 30 min. Specific activities of fatty acid methyl esters were determined as in Figure 2.

chromatography the pattern seen after TPIA treatment was quite different than that seen in the control tissue (Figure 2). Pretreatment with TPIA led to a striking decrease in the specific activities of all the long-chain polyunsaturated fatty acids.

As previously reported, aldosterone pretreatment led to a stimulation of precursor incorporation into phospholipid fatty acids and to an increase in the specific activity of a number of longer chain polyunsaturated fatty acids (Goodman et al., 1971, 1975). However, when bladders were pretreated with TPIA and then treated with aldosterone, the hormone did not cause either an increase in total fatty acid labeling (Table III) or in the pattern of specific activities (Figure 3). Additionally, a 2-hr pretreatment of bladders

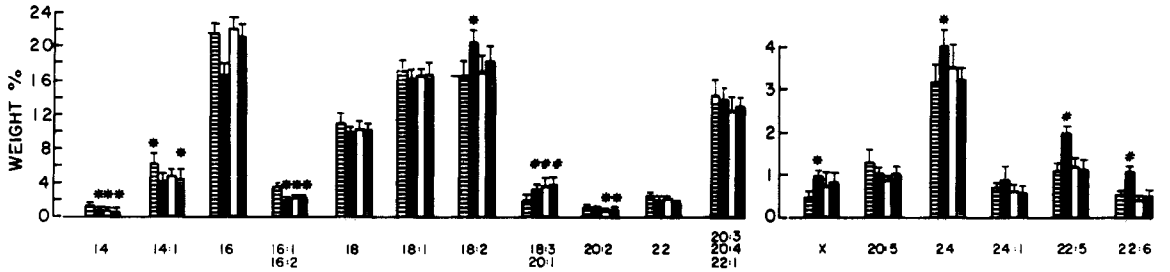

FIGURE 4: Fatty acid composition (weight percent) of toad bladder phospholipids in the presence and absence of TPIA. TPIA (2 m*M*, mucosa) was added 2 hr before a 6-hr aldosterone (10^{-7} *M*) incubation [(▤) control; (■) aldosterone; (□) TPIA; (▨) TPIA + aldosterone]. Values are the mean plus the standard deviation of six separate determinations; (*) $P < 0.05$; (#) $P < 0.02$.

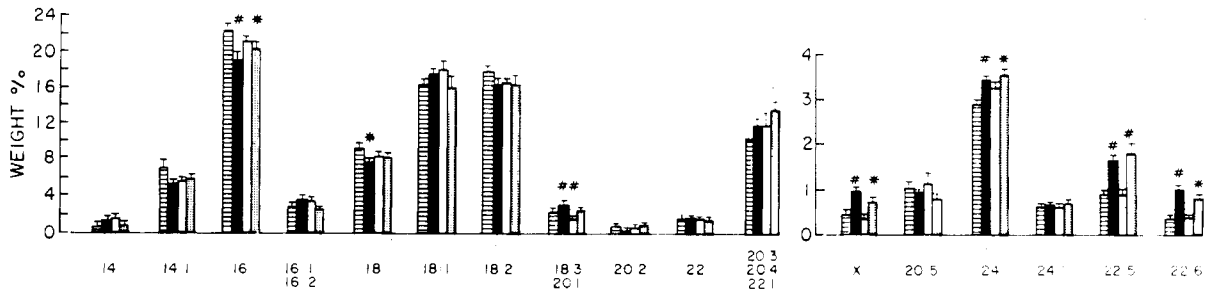

FIGURE 5: Fatty acid composition (weight percent) of toad bladder phospholipids in the presence and absence of amiloride. Amiloride (0.05 m*M*, mucosa) was added 2 hr before a 6-hr aldosterone (10^{-7} *M*) incubation [(▤) control; (■) aldosterone; (□) amiloride; (▨) amiloride + aldosterone]. Values are the mean plus the standard deviation of three separate determinations; (*) $P < 0.05$; (#) $P < 0.02$.

with 2 m*M* mucosal TPIA before a 6-hr exposure to aldosterone led to an inhibition of the usual aldosterone-induced increase in weight percentage of several phospholipid long-chain polyunsaturated acids (Figure 4). It is of interest that treatment of the tissue with mucosal TPIA alone for 6 hr caused little change in the weight percentage of phospholipid fatty acids (Figure 4).

Effects of Amiloride on Basal and Aldosterone-Stimulated Lipid Metabolism. Our data, as well as those of Ehrlich and Crabbé (1968), showed that the addition of 0.05 m*M* amiloride to the mucosal medium leads to a nearly total inhibition of basal as well as vasopressin- and aldosterone-stimulated Na^+ transport. When amiloride was added to the mucosal medium and [^{14}C]acetate incorporation measured, there was a 20% increase in total acetate incorporation (Table III); aldosterone treatment caused an additional 12% increase in incorporation (Table III). Aldosterone induced a change in the specific labeling pattern of phospholipid fatty acids in the presence as well as absence of amiloride (Figure 3). As shown in Figure 2, amiloride pretreatment caused little change in the specific activities of labeled phospholipid fatty acids compared to control tissue. When amiloride-pretreated tissue was exposed to aldosterone for 6 hr there was the same increase in weight percentage of long-chain polyunsaturated fatty acids in phospholipids as seen in tissue treated with aldosterone alone (Figure 5).

Reversal of Amiloride and TPIA Effects on Na^+ Transport. In view of the fact that both TPIA and amiloride blocked aldosterone-stimulated Na^+ transport but only TPIA blocked the aldosterone-induced changes in lipid metabolism, it was of interest to determine the rapidity with which Na^+ transport increased in aldosterone-treated bladders after removal of the inhibitors. Removal of amiloride from the mucosal bath after 3 hr of aldosterone treatment led to an immediate rise in short-circuit current to the level expected of the aldosterone-treated state. In contrast, when TPIA was removed from the mucosal solution after the bladder had been exposed to aldosterone for 3 hr there was no immediate rise in short-circuit current, but the short-circuit current did rise in approximately 60 min (Figure 1), the usual lag period between aldosterone addition and change in Na^+ transport.

Discussion

The present results lend support to the hypothesis that a change in the fatty acid composition of membrane phospholipids is an integral part of the response of the toad bladder to the steroid hormone, aldosterone. Since aldosterone stimulates acetate incorporation into phospholipid fatty acids and increases the weight percentage of long-chain polyunsaturated fatty acids in phospholipids in the presence of amiloride (Figures 3 and 5), the aldosterone-induced changes in lipid metabolism are not secondary to a change in Na^+ transport.

The results obtained with the inhibitor TPIA support the concept that a change in membrane lipid composition is a direct effect of aldosterone and that the aldosterone-induced change in lipid metabolism is a necessary step in the action of aldosterone on transcellular Na^+ transport. It is significant that 2 m*M* TPIA added to the mucosal surface of the bladder did not alter the effect of vasopressin upon either Na^+ transport or bulk water flow down an osmotic gradient (Table I) even though it blocked completely the aldosterone-induced increase in Na^+ transport (Figure 1).

The action of TPIA in blocking the aldosterone response appears to be relatively specific to lipid metabolism. TPIA did not alter the incorporation of labeled leucine or uridine into protein or RNA, respectively, in the presence or absence of aldosterone (Table II). It did, however, block the increase in both weight percentage of membrane phospholipid polyunsaturated fatty acids and [^{14}C]acetate incorporation into lipid usually seen after aldosterone action (Figures 3 and 4) (Goodman et al., 1975). From the work of

others it is known that TPIA inhibits acetyl-CoA carboxylase in vitro by competitively binding to the isocitrate-activating site (Maragoudakis, 1970; Maragoudakis and Hankin, 1971). The drug, however, does not inhibit a number of glycolytic, citric acid cycle, and fatty acid synthesis enzymes (Maragoudakis, 1969). Results from rat mammary gland cell culture also indicate an effect of TPIA on acetyl-CoA carboxylase; although production of $[^{14}C]CO_2$ and labeling of nonlipid material from [^{14}C]acetate remains unchanged, lipogenesis is greatly reduced (Maragoudakis, 1971). Long-term feeding experiments in vivo, however, indicate that TPIA has other effects. Altered hepatic enzyme levels (Schacht and Granzer, 1970), hepatomegaly (Beckett et al., 1972), microbody proliferation (Reddy et al., 1973), and lowered plasma triglyceride and cholesterol levels (Dujovne et al., 1970; Cenedella, 1971) have been observed. In the current study neither gross RNA nor protein synthesis nor the response to vasopressin appeared to be altered by TPIA. Fatty acid synthesis, as measured by [^{14}C]acetate incorporation in lipid, was, however, reduced 30%. Thus, in the present short-term in vitro incubations TPIA is probably acting at the level of acetyl-CoA carboxylase, the first committed step in fatty acid biosynthesis. Since the most striking effect of TPIA in control tissue was a reduction in chain elongation it is possible that TPIA has a direct effect on chain elongation or an indirect effect mediated by a reduction in the concentration of malonyl-CoA, the product of acetyl-CoA carboxylase and one of the substrates for chain elongation. Our data raise the possibility that the K_m for the participation of malonyl-CoA in chain elongation is higher than for de novo fatty acid synthesis.

Although TPIA greatly reduced the incorporation of [^{14}C]acetate into phospholipid long-chain polyunsaturated fatty acids it caused little change in the weight percentage of the long-chain polyunsaturated fatty acids in this tissue. However, from previous results (Goodman et al., 1971, 1975), it is apparent that when aldosterone acts to increase lipid incorporation into phospholipids it also increases the rate of phospholipid deacylation. Thus, these data and the present results suggest a coupling between deacylation and reacylation in membrane phospholipids, and when the availability of substrate for reacylation decreases, deacylation is reduced.

Two important questions remain from our present results. These are: (1) the mechanism by which aldosterone alters lipid metabolism; and (2) the relationship between the aldosterone-induced change in lipid metabolism and the change in Na^+ transport. With regard to the second question, at least two alternatives are possible. Either the changes in membrane lipids are primary and act to alter the environment and thereby the activity of a membrane-bound enzyme(s) or transport carrier(s), or the primary effect of aldosterone is to increase the synthesis of a membrane protein which requires a new lipid environment before it can become a functional unit in the membrane.

Of these two alternatives the former is more attractive. There are precedents for the fact that changes in the fluidity of a membrane can alter the activity of membrane proteins (Grisham and Barnett, 1973; Kimelberg and Papahadjopoulos, 1974). The significant increase in chain length, and particularly the degree of unsaturation of membrane fatty acids seen after aldosterone treatment (Figure 4), should lead to a significant change in membrane fluidity. Such a change in membrane lipid composition could account for the fact that aldosterone not only increases Na^+ transport but also alters the sensitivity of this tissue to the toxic effects of hyperbaric oxygen (Allen et al., 1973) and enhances its response to vasopressin (Handler et al., 1969; Goodman et al., 1969). On the other hand, if the primary effect of aldosterone were that of controlling the synthesis of a membrane protein the amount of this newly synthesized protein would be extremely small, and even if a change in lipid composition were necessary for its incorporation into the membrane the magnitude of the change in lipid composition necessary to encompass this new protein would be quite small and not of the magnitude seen in our studies. Conversely, if one accepts the possibility that the biochemical change of major physiological importance is that in membrane lipids it is then possible to propose that inhibitors of protein and RNA metabolism block the action of aldosterone on Na^+ transport by an inhibition of the synthesis of a key enzyme(s) in fatty acid metabolism and not by an inhibition of the synthesis of a membrane protein.

If an inhibition of the synthesis of an enzyme functioning in fatty acid metabolism were the basis of the effects of inhibitors of protein and RNA synthesis upon aldosterone-induced Na^+ transport, then treatment of the toad bladder with such inhibitors should block the aldosterone-induced increase in the weight percentage of phospholipid polyunsaturated fatty acids. Experiments, using the inhibitor cordycepin, show this to be the case (Lien et al., 1975). This inhibitor of messenger RNA synthesis blocked both the aldosterone-induced increase in Na^+ transport (Chu and Edelman, 1972) and the aldosterone-induced increase in weight percentage of phospholipid long-chain polyunsaturated fatty acids (Lien et al., 1975).

Our present data indicate that the aldosterone-induced change in membrane lipid composition is apparently a necessary step in the sequence of events which lead to a change in the rate of transcellular Na^+ transport. They raise the distinct possibility that some hormones can alter specific tissue functions by altering the lipid environment of membrane proteins and thereby alter either the catalytic activity of individual proteins and/or the number of such proteins in the membrane. This hypothesis represents a new model to account for the action of some hormones, particularly those steroids which alter membrane ion transport, i.e., aldosterone and vitamin D (Goodman et al., 1972).

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Initial Membrane Reaction in the Biosynthesis of Peptidoglycan. Spin-Labeled Intermediates as Receptors for Vancomycin and Ristocetin†

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ABSTRACT: Phospho-*N*-acetylmuramyl-pentapeptide translocase (UDP-MurNAc-Ala-DGlu-Lys-DAla-DAla:undecaprenyl phosphate, phospho-MurNAc-pentapeptide transferase) catalyzes the initial membrane reaction in the biosynthesis of peptidoglycan. The spin-labeled nucleotide, UDP-MurNAc-Ala-DGlu-Lys(N^{ϵ}-2,2,5,5-tetramethyl-*N*-oxylpyrroline-3-carbonyl)-DAla-DAla, was used as a substrate by this enzyme for the synthesis of membrane-associated undecaprenyl-diphosphate-MurNAc-Ala-DGlu-Lys(N^{ϵ}-Tempyo)-DAla-DAla. The spin-labeled substrate and product complex with the antibiotics vancomycin and ristocetin. The association constants for the spin-labeled nucleotide are 6.2×10^5 and 6.2×10^4 M^{-1} for vancomycin and ristocetin, respectively. The association constants for the spin-labeled lipid intermediate are 3.0×10^4 and 2.1×10^4 M^{-1} for vancomycin and ristocetin, respectively. These results indicate that the acyl-DAla-DAla termini of membrane-associated spin-labeled undecaprenyl-diphosphate-MurNAc-pentapeptide are accessible to vancomycin and ristocetin and that the association constants are smaller than those determined for the corresponding antibiotic spin-labeled UDP-MurNAc-pentapeptide complexes.

The biosynthesis of peptidoglycan is catalyzed by a series of membrane-associated enzymes that utilize two nucleotide-activated precursors, UDP-*N*-acetylglucosamine and UDP-*N*-acetylmuramyl-pentapeptide (Ghuysen and Shockman, 1973). The initial enzyme of this series, phospho-MurNAc-pentapeptide[1] translocase, catalyzes the transfer of phospho-MurNAc-pentapeptide from UDP-MurNAc-pentapeptide to undecaprenyl phosphate with the formation of undecaprenyl-diphosphate-MurNAc-pentapeptide (Neuhaus, 1971). This reaction results in the transfer of a precursor from the cytoplasm to the membrane. A reporter group that reflects the microenvironment in each of these phases would be useful in probing this reaction.

Spin-labels provide sensitive probes to study intermediates in enzyme-catalyzed reactions. The electron spin resonance (ESR) spectrum of the spin-label is a function of the motion that the probe experiences and the polarity of the solvent surrounding the probe. Thus, information about the mobility of the spin-label and its microenvironment can be deduced from its spectrum. It is the purpose of this paper to report the synthesis of spin-labeled UDP-MurNAc-pentapeptide and membrane-associated spin-labeled undecaprenyl-diphosphate-MurNAc-pentapeptide and to utilize these compounds as receptors for the antibiotics, vancomycin and ristocetin. These experiments indicate that the acyl-

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[1] Unless stated, all abbreviations of residues denote the L configuration. The omission of the hyphen, i.e., -DAla- for -D-Ala, conforms with the suggestion cited in *Biochemistry 5,* 2485 (1966). Although not stated, all D-glutamic acid residues are linked through the γ-carboxyl group to the α-amino group of the diamino acid. In UDP-MurNAc-pentapeptide the residues are numbered as follows: UDP-MurNAc-Ala^1-DGlu^2-Lys^3-DAla^4-DAla^5. Abbreviations used are: Tempyo, 2,2,5,5-tetramethyl-*N*-oxylpyrroline-3-carbonyl-; MurNAc, *N*-acetylmuramyl; GlcNAc, *N*-acetylglucosamine; *m*Dap, *meso*-α,ε-diaminopimelic acid; UDP, uridine diphosphate.