Hydrogen–Deuterium Exchange in Nucleosides and Nucleotides. A Mechanism for Exchange of the Exocyclic Amino Hydrogens of Adenosine†

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ABSTRACT: The pH dependence of the apparent first-order rate constant for the exchange of the exocyclic amino hydrogens of adenosine with deuterium from the solvent was measured by stopped-flow ultraviolet spectroscopy. This dependence shows acid catalysis, base catalysis, and spontaneous exchange at neutral pH values. A study of the effect of several buffers on the rates of exchange reveals both general acid and general base catalytic behavior for the exchange process. We propose a general mechanism for the exchange which requires N-1 protonated adenosine as an intermediate for the acid-catalyzed exchange and amidine anion for the base-catalyzed exchange. In both cases the rate-limiting step is the base-catalyzed abstraction of a proton from the exocyclic amino moiety. Evaluation of the rate constants predicts the equilibrium for the exocyclic amino/imino tautomers to be 6.3×10^3:1.

The use of static and stopped-flow ultraviolet spectrophotometry to measure directly the rate of hydrogen exchange to the exocyclic amino nitrogen of adenosine and adenosine analogs was introduced in the preceding paper in this series (Cross, 1975). This method measures the time dependence of the change in absorbance in the 260-nm region which results from a blue shift of the absorbance band of adenine upon replacement of hydrogen by deuterium at the N-6 nitrogen.

McConnell and Seawell (1972) and McConnell (1974) have used nuclear magnetic resonance (NMR) to study the pH dependence of the exchange of hydrogens at the N-6 moieties of AMP and 3′,5′-cyclic adenosine monophosphate (cAMP). The first study shows a second-order dependence of the relaxation process on AMP concentration which the authors attribute to intermolecular catalysis of exchange involving the ribose–phosphate moiety. The intermolecular catalysis was confirmed by Raszka (1974) and by Cross (1974, 1975) when it was shown that ribose phosphate and inorganic phosphate produced the same second-order dependence with analogs of AMP lacking the phosphate moiety. This phosphate catalysis of the exchange together with the observation of imidazole catalysis of the exchange of hydrogens to cAMP (McConnell, 1974) suggests that exchange of hydrogens to adenosine would show characteristics of general acid-base catalysis.

Although it has been suggested that the N-1 protonated form of adenosine is required for exchange at neutral and acid pH values in the absence of buffer (McConnell, 1974), there has been no detailed study of the role of buffer catalysis of the exchange process. We will show that the exchange of hydrogens at the exocyclic amino group of adenosine is general acid and general base catalyzed. The salient features of the proposed mechanism which accounts for the pH dependence of the apparent first-order rate constant for exchange include rate determining proton abstraction from N-6 of the protonated adenosine in the low and neutral pH regions and rate-determining proton abstraction from N-6 of neutral adenosine in the high pH region.

Experimental Procedure

Adenosine, AMP, glycylglycine hydrochloride, imidazole, and deuterium oxide were purchased from Sigma Chemical Company. Sodium phosphate, sodium chloride, sodium hydroxide, and hydrochloric acid, all analytical reagent grade, were products of Mallinckrodt Chemical Works. PCR, Incorporated was the supplier of 2,2,2-trifluoroethylamine hydrochloride.

A pH meter with an accuracy of ±0.2 pH unit was used to measure pH values; pD values were determined by the approximation of Glasoe and Long (1960), $pD = pH_{meter} + 0.4$. The rate of hydrogen exchange was determined from the time-dependent absorbance change at 285–293 nm measured in a Durrum-Gibson stopped-flow spectrophotometer equipped with thermostated cells of 0.54-cm path length, as described previously (Cross, 1975). The dead time of the instrument was measured using the reaction of ferric nitrate with potassium thiocyanate in 0.1 N H_2SO_4 and was found to be 3–4 msec. Data were collected over times which included at least 3 half-lives of the reaction at 0.5- and 1.0-msec intervals using a Varian 620L computer. From 7 to 13 individual rate measurements were averaged to obtain the final progress curve for each experiment. These averaged data were then used to determine the apparent first-order rate constant for the reaction in a plot of log $(A_t - A_\infty)$ against time where A_t is the absorbance at time t and A_∞ is the final value of the absorbance obtained at times where no further changes were apparent. Unless otherwise noted the exchange process was initiated by the mixing of equal volumes of adenosine or adenosine analog dissolved in deuterium oxide solvent with protium oxide using the stopped-flow drive syringes. In experiments in which buffer concentration or pH was varied, both syringes contained the same amount of varied constituent. All experiments were performed in 0.01 M sodium chloride and the temperature was maintained at 20° unless otherwise noted.

Results

Dependence of Hydrogen Exchange on pH. The rate of exchange of the exocyclic amino hydrogens of adenosine was measured over a pH range from 2.5 to 10. The depen-

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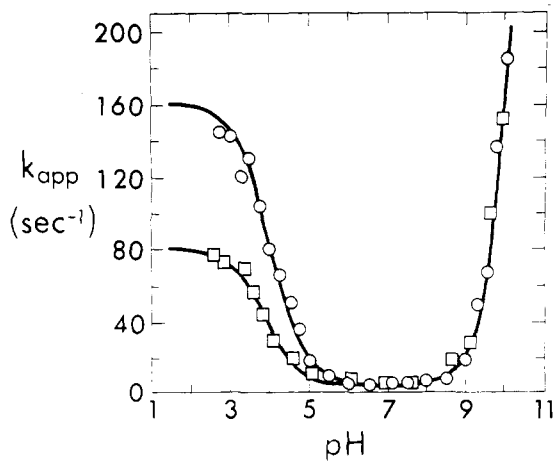

FIGURE 1: Dependence of the apparent first-order rate constant (k_{app}) for the exchange of hydrogens at the exocyclic amino moiety of adenosine on pH. The data shown by the circles represent rate constants calculated from the exchange of 99.5% deuterated adenosine with protium oxide and the squares show data for the exchange of protiated adenosine with deuterium oxide, the final isotope concentration in both experiments being approximately 50%. The theoretical lines drawn through the data are calculated from eq 1 using the values given in the text.

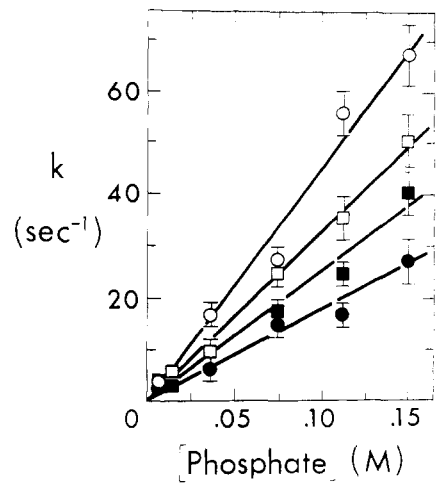

FIGURE 2: Dependence of the apparent first-order rate constant for exchange of the exocyclic amino hydrogens of adenosine on sodium phosphate concentration. The pH (pD) values are: 7.6 (●), 7.0 (■), 6.5 (○), and 6.0 (□).

dence of the apparent first-order rate constant for exchange on pH is shown in Figure 1. These data show an apparent pK_a shift in the low pH region, the magnitude of which is the same as that observed for other weak acids (Laughton and Robertson, 1969). The data in Figure 1 also show a relatively large kinetic isotope effect only in the low pH region which indicates that the rate of exchange of deuterium from the exocyclic amino moiety is twofold greater than the rate of exchange of hydrogen. The final solvent composition for both directions of exchange is a 50% mixture of D_2O and H_2O. The lines drawn through the data in Figure 1 represent a best fit of the following empirical equation to the data points

$$k_{app} = \frac{k_H a_H}{a_H + K_a} + \left(k_0 + \frac{k_{OH}K_w}{a_H}\right)\left(\frac{K_a}{a_H + K_a}\right) \quad (1)$$

where k_{app} is the apparent first-order rate constant measured for the exchange, a_H is hydrogen ion activity, K_a is the acid dissociation constant for adenosine, k_H is a pH-independent acid rate constant, k_0 is the spontaneous rate constant obtained from the pH-independent rate at neutral pH, and k_{OH} is the rate constant for hydroxide ion catalysis obtained by extrapolation of the logarithm of the apparent first-order rate constant to 1 M hydroxide ion. The values of the constants required to fit the two sets of data in Figure 1 are shown in Table I.

Buffer Catalysis of Hydrogen Exchange. Phosphate, imidazole, glycylglycine, and trifluoroethylamine were examined for general acid and general base catalysis of the rate of exchange of hydrogens to the exocyclic amino group of adenosine. The first-order rate constants for exchange were measured at buffer concentrations varying from 0 to 0.15 M at 0.15 ionic strength and at a minimum of three pH values which were centered around the pK_a of the buffer being used. Plots of the apparent first-order rate constants against buffer concentrations were linear in every case and were similar to that shown in Figure 2 for phosphate catalysis. Rate constants independent of buffer concentration, k', were obtained from the slopes of the linear least-squares fits

Table I: Values of the Constants in Eq 1 which were Used for a Best Fit of the Data in Figure 1.

Experiment					
Syringe 1	Syringe 2	pK_a	k_{OH} (M^{-1} sec^{-1})	k_H (M^{-1} sec^{-1})	k_0 (sec^{-1})
Ado + D_2O	H_2O	4.0	$1.1 \pm 0.2 \times 10^7$	160 ± 20	4.8 ± 0.5
Ado + H_2O	D_2O	3.8	$1.0 \pm 0.4 \times 10^7$	78 ± 8	4.6 ± 0.6

to the data. A second plot of k' against the fraction free acid is linear for each buffer and the catalytic rate constants, k_b and k_a, for each buffer were determined from the intercepts and slopes of the plots, respectively. These values are listed in Table II along with the values for water determined from the data in Table I. The values of the catalytic rate constants in Table II were used to produce the Bronsted plot shown in Figure 3. A linear least-squares fit of the data in Figure 3 resulted in slopes of -0.41 ± 0.08 for the acid catalysis line and 0.56 ± 0.10 for the base catalysis line.

Temperature Dependence of Hydrogen Exchange. Figure 4 shows the temperature dependence of the apparent first-order rate constant for the exchanges of deuterated adenosine and AMP with hydrogen from the solvent at pH(D) = 7. For this reaction the energies of activation are 10.5 ± 0.5 and 10.9 ± 0.5 kcal mol^{-1} for adenosine and AMP, respectively.

Discussion

The fit of eq 1 to the pH dependence of the apparent first-order rate constant for deuterium/hydrogen exchange in Figure 1 indicates that the exchange is characterized by three distinct rate constants. The scheme shown in Figure 5 is proposed to account for the pH dependence of the exchange rate of the exocyclic amino hydrogens of adenosine. In this scheme exchange occurs through two acid–base catalyzed processes: (1) an exchange which requires the N-1 protonated species of adenosine to be intermediate to the amino and imino tautomers and (2) an exchange which requires the formation of the amidine anion followed by reprotonation of the exocyclic amino group. Using the scheme

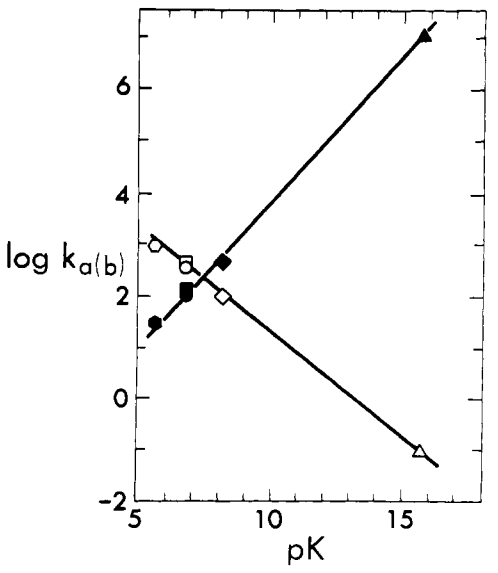

FIGURE 3: Bronsted plots for the acid and base catalysis of hydrogen exchange at the exocyclic amino group of adenosine. Acid and base catalytic constants (k_a and k_b) are determined as described in the text and are indicated by open and solid symbols, respectively. The catalytic species are: water (△), phosphate (○), imidazole (□), glycylglycine (◇), and trifluoroethylamine (○). The slopes of the least-squares fit to the data for acid and base catalysis are −0.41 ± 0.08 and 0.56 ± 0.10, respectively.

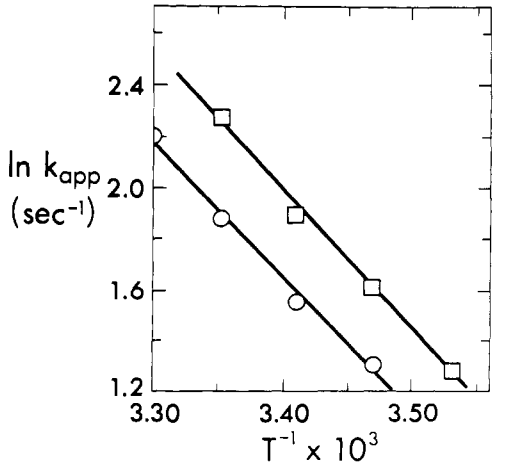

FIGURE 4: Temperature dependence of the apparent first-order rate constant for the exchange of hydrogens at the exocyclic amino moiety of adenosine. Circles are data points acquired using adenosine and squares are from hydrogen exchange measurements using AMP. Both compounds were preequilibrated in D_2O and mixed with an equal volume of H_2O in the stopped-flow apparatus to a final concentration of 2.2 m*M*. Temperature is given in degrees Kelvin.

in Figure 5 with the assumption that the rate of exchange of the second hydrogen is the same as the rate of exchange of the first, the apparent first-order rate constant (k_{app}) will be given by the rate equation:

$$k_{app} = \sum_i \left(\frac{[B_i]}{1 + a_H/K_a}\right)\left(k_3^i + \frac{a_H k_2^i}{K_a}\right) \qquad (2)$$

where $[B_i]$ is the concentration of any base, i designates the conjugate base of the acid–base pair, K_a is the acid dissociation constant for adenosine, and the sum is taken over all the acid–base species in the system.

At high pH values (pH 8–10) and in the absence of buffers eq 2 reduces to $k_{app} = k_3^{OH}[OH^-]$ and the value for k_3^{OH} becomes identical with k_{OH} (1.1×10^7 M^{-1} sec^{-1} in Table I). This value fits on the line for general base catalysis in the Bronsted plot in Figure 2. Thus, we propose that the exchange of hydrogens can occur through the general base catalyzed formation of an amidine anion in which the reprotonation, k_{-3}, step is diffusion controlled. This mechanism is the same as that proposed by McConnell and Seawell (1972) for adenine nucleotides, by Miller and Klotz (1973) and Berger et al. (1959) for base-catalyzed exchange in amides, and also for the base-catalyzed exchange of hydrogen in ketones (Bruice and Benkovic, 1966).

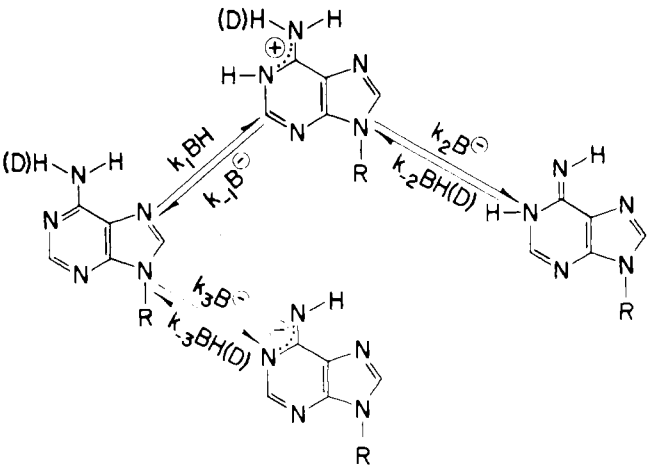

FIGURE 5: A scheme for the exchange of hydrogens at the exocyclic amino group of adenosine.

Table II: Catalytic Rate Constants for the Exchange of the Deuterated Exocyclic Amino Moiety with Protium from the Solvent.

	pK_a	k_a (M^{-1} sec^{-1})	k_b (M^{-1} sec^{-1})
Trifluoroethylamine	5.7	631	32
Imidazole	6.8	413	98
Phosphate	6.8	352	120
Glycylglycine	8.2	88	428

At low pH values and in the absence of buffer we propose the exchange to occur predominantly through the N-1 protonated species of adenosine and in this case eq 2 reduces to:

$$k_{app} = a_H k_2^{H_2O}/(a_H + K_a) \qquad (3)$$

since H_3O^+ and H_2O are the respective conjugate acid–base pair. The pH dependence of the apparent first-order rate constant in Figure 1 shows that the hydrogen exchange rate increases in proportion to the increase in the concentration of the N-1 protonated species and reaches a plateau of 160 ± 20 sec^{-1} at pH values where the adenine moiety is fully protonated. This value agrees with that determined from a relaxation process measured by NMR with 3′,5′-cyclic adenosine monophosphate which was attributed to decreased $-NH_2$ rotation about the bond to the C-6 atom (McConnell, 1974). Our mechanism is supported by the agreement of the known pK_a for N-1 protonation of adenosine, 3.65 (Wolfenden, 1969), with the apparent pK_a for the exchange of deuterium (4.0) and hydrogen (3.8) determined from the data. In addition, Cross (1975) reports that

the rate of exchange of the N-6 hydrogens of N^1-methyladenosine, an analog of the protonated form of adenosine, is much more rapid at neutral pH values than the rates observed here. This observation is in agreement with this mechanism which predicts the rate of exchange to be proportional to the concentration of N-1 protonated adenosine. Jencks (1969) has noted that the most common reason for the occurrence of an inverse isotope effect is that hydrogen transfer occurs at a transition state which differs from the transition state of the reaction, a situation which applies to diffusion controlled reactions. Therefore, the inverse isotope effect, $k_2^H/k_2^D = 0.5$, observed in the diffusion-controlled exchange, which is acid catalyzed, is consistent with the mechanism proposed for this exchange.

The dependence of the rate constants on buffer concentration and pH reveals both general acid and general base catalysis. According to the mechanism for general base catalysis the conjugate bases of each buffer abstract a proton from N-6 in the rate-determining step. The general acid catalytic behavior, on the other hand, can be accounted for by two kinetically equivalent mechanisms: (1) in the rate-determining step the conjugate acid of the buffer donates a proton to N-1 of adenosine while a base species abstracts a proton from N-6; (2) a mechanism which is parallel to that proposed in eq 3 above for specific acid catalysis with the exception that the conjugate base of the buffer abstracts the proton from N-6 in the rate-determining step. We favor the second of these mechanisms because kinetically a second-order term in buffer concentration would be expected for mechanism 1 and none was detectable. The rate constants for general acid catalysis fall on a single line of the Bronsted plot in Figure 2 except for hydronium ion. Although not shown in the plot, this datum falls below the line because the sum of the rates of proton abstraction from N-1 and N-6 of the protonated species by water is diffusion limited.

At neutral pH values k_{app} in Figure 1 is independent of pH and is identical k_0 in eq 1. From eq 2

$$k_{app} = k_0 = \frac{K_a k_3^{H_2O}[H_2O] + a_H k_2^{OH}[OH^-]}{a_H + K_a} \quad (4)$$

At neutral pH the acid dissociation constant for adenosine, K_a, is much larger than a_H, and since $a_H[OH^-] = K_w$ it is readily seen that k_{app} will then be independent of pH. An estimate of the contribution to k_0 by general base catalysis is obtained by extrapolating the Bronsted line to -1.7, the pK_a of water, giving $k_3 = 10^{-2.7}\ M^{-1}\ sec^{-1}$. This small value indicates that the major contribution to k_0 at neutral pH values probably occurs through the general acid mechanism where water is the general acid. To support this contention, k_0 falls on the Bronsted line for general acid catalysis.

According to eq 2 and using H_3O^+ and H_2O as the conjugate acid–base pair, the first-order rate constant in the low pH region has the identity:

$$k_{app} = \left(\frac{a_H}{a_H + K_a}\right)(k_2^{H_2O}[H_2O] + k_2^{OH}[OH^-]) \quad (5)$$

Using the data for the exchange of deuterated adenosine with H_2O in the low pH region and in the absence of buffer, a linear plot of k_{app}/H_2O against $a_H/a_H + K_a$ is obtained. The slope of this plot gives $k_2^{H_2O} = 2.9 \pm 0.8\ M^{-1}\ sec^{-1}$.

An apparent pK_a for the exocyclic imino tautomer of adenosine is calculated to be approximately 7.8 with the assumptions that the rate of protonation, k_{-2}, is diffusion controlled or $10^{10}\ M^{-1}\ sec^{-1}$ and that k_H in Table I is the rate of dissociation of the protonated adenosine to the imino tautomer. This value is comparable to a $pK_a = 8.25$ (Macon and Wolfenden, 1968) for the protonation of the exocyclic nitrogen of N^1-methyladenosine, an analog of the imino tautomer of adenosine. The ratio of the dissociation constants for the exocyclic amino and exocyclic imino isomers of adenosine gives a value of 6.3×10^3 for the amino/imino tautomeric ratio. This value is comparable to a ratio of 4×10^4 estimated by Wolfenden (1969) from the pK_a values of adenosine analogs.

The exchange of hydrogen to some polynucleotides and RNA is slow enough to be measured using column chromatography (Printz and von Hippel, 1968; McConnell and von Hippel, 1970; Hanson, 1971; Englander et al., 1972a). The slow exchange rates were variously ascribed to the effects of hydrogen bonding and to the unavailability of the exchangeable hydrogens to the medium. Englander et al. (1972b) noted that some of the exposed hydrogens in NH_2 groups of polynucleotides and tRNA exchanged slowly. The formation of Watson–Crick hydrogen bonds stabilizes the adenine base in the exocyclic amino tautomer which will decrease the pK_a for N-1 protonation and increase the pK_a for amidine anion formation. According to the mechanism for hydrogen exchange both of these pK_a changes will decrease the rate of exchange of the amino hydrogen involved in hydrogen bonding and of the nonbonded amino hydrogen which is exposed to solvent.

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Added in Proof

In a recent paper Teitelbaum and Englander (1975) conclude that the very slow exchange of the exocyclic amino hydrogens of adenine in polynucleotides is due to the chemistry of exchange using a mechanism essentially the same as that proposed here rather than due to a change in the relative exposure of the hydrogens to the exchanging medium.

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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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