TABLE II. U-21221 on Specific Activity of Plasma Free Fatty Acids.

Treatment	No.	Dose (mg/kg)	Blood sugar (mg%)	FFA	
				μE/l*	CPM/μE†
Controls	5	—	82	952	.70
U-21221	5	.5	76	240‡	1.80‡

* Microequivalents/liter.
† Counts per minute/microequivalent.
‡ $P < .01$.

duced by insulin. Since insulin has an effect on FFA(2), it was of interest to study effects of U-21221 on FFA. Data reported here show that the isoxazole also depresses FFA. Observation showing that U-21221 does not alter oxidation of palmitate-1-C^{14} and increased specific activity of circulating FFA after injection of labeled palmitate suggests that the mechanism of U-21221 depression of FFA was due to decreased release from the depots.

The finding that FFA were depressed prior to change in blood sugar suggests that blood sugar change might be due to a compensatory increased glucose utilization due to reduction of FFA as an energy source or removal of the inhibitory effect of FFA on glucose utilization(3,4). However, this is probably an over simplification of the mechanism of action of U-21221 on blood sugar since FFA were depressed in barbiturate-treated intact rats following treatment with isoxazole without a decrease in blood sugar. These results suggest that there is either no relationship of the FFA and blood sugar effects of U-21221 or that barbiturates and evisceration are able to specifically block the action of U-21221 on blood sugar.

Summary. 3,5-Dimethylisoxazole was shown to decrease FFA, increase specific activity of plasma FFA after injection of labeled palmitate but to have no effect on palmitate oxidation. These observations were interpreted to mean that U-21221 lowered FFA as a result of decreased release from depots. Depression of FFA occurred prior to the fall in blood sugar in intact rats treated with U-21221. The isoxazole caused a depression of FFA in barbiturate-treated intact rats and in eviscerated rats without producing depression of blood sugar. These results were interpreted to mean that either the effects of U-21221 on FFA and blood sugar were independent of each other or that barbiturates and evisceration can specifically block action of U-21221 on blood sugar.

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Action of Aldosterone and Ouabain on O_2 Consumption and Short-Circuit Current of Toad Bladder.* (29889)

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It has been established that aldosterone is capable of stimulating active sodium (Na^+) transport across the isolated urinary bladder of the toad(1-4). The exact mechanism

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whereby this effect is produced is still incompletely understood.

Reports by various investigators(5-9), however, have now defined many of the basic biochemical and physiological properties of the transport mechanism in the toad bladder, and recently Edelman *et al*(3) and Crabbe(10) presented evidence indicating that aldosterone may be stimulating active Na^+ transport in this tissue by inducing *de novo* synthesis of enzymes involved in supplying energy for cation transport.

It is generally recognized that a portion of the oxygen uptake of cells is related to the expenditure of metabolic energy involved in the processes of actively transporting Na^+ (11). While recent reports have defined some of the metabolic requirements and functions of the toad bladder(8,9), the exact nature of the link between the energy-yielding processes (metabolism) of this tissue and the active transport of Na^+ is unknown. Also, the manner in which these energy-yielding processes are influenced by agents such as aldosterone which can affect Na^+ transport remains to be explained. Therefore, we considered it desirable to determine whether or not aldosterone had any effect on the oxidative metabolism of bladder tissue in concentrations known to stimulate Na^+ transport.

Moreover, since ouabain is recognized as an inhibitor of active Na^+ transport in a variety of cells and tissues, we also determined the effects of the glycoside on the O_2 consumption of the isolated bladder. Separate experiments were also performed to characterize the effects of these two steroids on the short circuit current of the isolated bladder.

Methods. The bladders used in all experiments were obtained from female toads (*Bufo marinus*) weighing 250-300 g. Upon receiving the animals from the supplier,† they were divided into 2 groups. Group I was immersed in small tanks containing distilled water. These animals were used 3-7 days following this treatment. This procedure is known to increase the sensitivity of the bladder to the exogenous effects of aldosterone(1). Group II was kept in a large metal cage containing mud.

The hemibladders obtained from Group I were always found to be markedly distended and generally had more of a "mucosal" appearance than bladders from the other groups. The bladders from Group II were also found to be distended, but were characterized by a clear, transparent appearance in contrast to the toads kept in distilled water.

Measurement of O_2 consumption. After pithing an animal, the hemibladders were removed and placed into an oxygenated toad Ringer solution for rinsing. The bladders were then transferred into petri dishes containing a phosphate buffered medium(9) of the following composition (mEq): NaCl, 111.4; KCl, 1.88; $CaCl_2$, 1.78; NaH_2PO_4, 0.1; Na_2HPO_4, 4.0. Modification of this basic medium was made as noted under the section on Results. The bladders were finely minced with scissors. This was done to avoid the tendency of intact bladders to curl and fold and hence possibly interfere with O_2 diffusion through the tissue. The minced bladders were blotted gently on filter paper to remove excess fluid, and portions were then placed into Warburg flasks containing 2.98-3.0 ml of the phosphate Ringer's. Drug and diluent effects were studied by appropriate additions to the flask media so as to keep the flask contents constant at 3.0 ml. The center wells contained 20% KOH. After the tissues were in the flasks, they were mounted on the recording manometers and placed in a constant temperature tank maintained at 37.5°C. Although the temperature is approximately 12°C higher than that used for the short-circuit current measurements, the higher O_2 consumption recorded permitted the detection of relatively small changes which were obscured at lower (25°C) values. The flasks were gassed for 10 minutes with 100% O_2 while being shaken during the temperature equilibration period. At the end of 10 minutes, the flask vents were sealed and the recording of O_2 uptake begun. The recording of the data was facilitated by the use of an automated device‡ which permitted continuous recording of O_2 uptake by the

† Tarpon Springs Zoo, Florida.

‡ Mechrolab, Inc., Mountain View, Calif.

tissues in μl O_2. Experiments were run for 2-5 hours. At the end of experiment, the tissues were removed from the flasks, lightly blotted and placed in tared weighing crucibles in a drying oven overnight at 110°C. Dry weights were obtained, and O_2 consumption plotted in μl O_2/mg dry weight *versus* time.

Measurement of short-circuit current. The apparatus and techniques used for measurement of the short-circuit current (scc) of the isolated bladders were of the basic design described by Ussing and Zerahn(12). The hemibladders were incubated for 18-24 hours after mounting in a drug-free bicarbonate frog-Ringer's solution (20 ml in each half-chamber) as described by Porter and Edelman(4). After this incubation period, the solutions were exchanged, and a glucose-fortified (10 mM) frog-Ringer's was substituted. One-half to one hour later, the drug being studied was added to the solution bathing the serosal side of one hemibladder, and drug diluent added to the other bladder which served as a simultaneous control. Active Na^+ transport, as measured by the short-circuit current, was continuously monitored by use of an automated servo device‡ which keeps the membrane potential at 0 mv while continuously plotting the shorting current in μa.

Results. O_2 uptake. a. *Aldosterone effects in normal media.* Fig. 1 illustrates the O_2 uptake of minced bladders from Group II toads. Over a 3-hour period, O_2 uptake gradually decreased in the controls. D-aldosterone (1.9×10^{-7} M) failed to significantly affect ($P>0.05$) O_2 uptake, although there is an indication that O_2 uptake in the steroid-treated bladders remains nearly linear over the 3-hour period of observation.

b. *Aldosterone and ouabain effects in glucose-enriched media.* Fig. 2 shows O_2 uptake of bladders from Group II toads in a medium fortified with glucose (10 mM). The control group was characterized by a more linear O_2 uptake than the untreated controls in a glucose-free media. Of interest is the finding that 1.9×10^{-7} M d-aldosterone tended to produce a stimulation of O_2 uptake relative to these controls, while 2.8×10^{-4} M ouabain tended to cause a decrease in O_2 uptake measured over a 5-hour period. However, the mean QO_2 values for these 2 steroid groups are not significantly different when compared with the controls at 5 hours.

The apparent tendency of glucose in the media to unmask the steroid effects prompted us to study the actions of aldosterone and ouabain on bladders taken from toads immersed in distilled water for 3-7 days (Group I) using a glucose-enriched media. Fig. 3 shows the results of these experiments. The untreated controls showed a non-linear O_2 uptake characterized by an initial decrease during the first 60-90 minutes, followed by a progressively increasing O_2 consumption thereafter. Aldosterone (1.9×10^{-7} M) produced a sustained increase in O_2 uptake of the tissue relative to the controls so that at the end of 5 hours, the mean O_2 consumption of the steroid-treated group was significantly greater ($P>0.01$) than the control group. There is also a suggestion that at 2.5-3.0 hours there is an increase in the rate of O_2 consumption compared to the rate seen during the first 2 hours.

Ouabain (2.8×10^{-4} M) tended to produce an increase in O_2 consumption in contrast to the untreated controls, although the mean value at 5 hours is not significantly different ($P>0.05$) than the value for the control group.

c. *Aldosterone and ouabain effects in a high K^+ media.* Experiments were performed to compare O_2 uptake changes seen in a high K^+ media (18.8 mEq) *versus* those seen in a normal K^+ media (1.88 mEq). Under these conditions, steroid effects on O_2 uptake are absent, the O_2 uptake of the aldosterone and ouabain treated groups being nearly identical to that shown by the untreated controls. There was no evidence that the aldosterone-treated bladders showed a more linear O_2 uptake than did the untreated bladders (as was seen in Fig. 1) where a normal (1.88 mEq) K^+ was used. However, O_2 uptake of the untreated tissues in the high K^+ media was significantly greater ($P<0.05$) than the value recorded in the untreated controls in a normal K^+ media when compared at 3 hours.

Short-circuit current (scc). a. *Effects of ouabain.* Addition of ouabain in concentra-

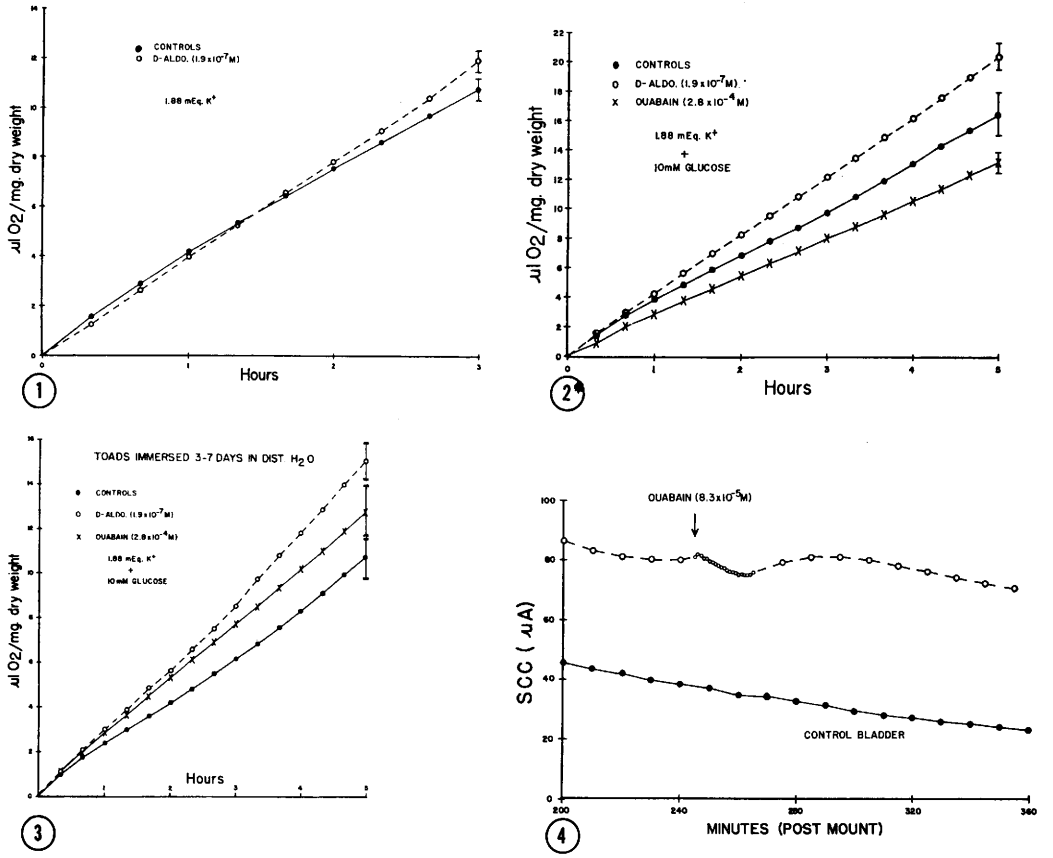

FIG. 1. Aldosterone on O_2 consumption of minced distended ("D") bladders from toads kept in mud (Group II toads). See *Methods* for full description of toad preconditioning and condition of bladder. Each point represents mean value of 5-7 experiments. SE of mean at 3 hr is indicated by vertical bars.

FIG. 2. Aldosterone and ouabain on O_2 consumption of minced bladders (Group II) in a glucose-fortified media. Each point represents mean value of 4 experiments.

FIG. 3. Aldosterone and ouabain on O_2 consumption of minced bladders from toads kept in distilled water for 3-7 days (Group I). A glucose-fortified media was used as in Fig. 3. Each point represents mean value of 5-7 experiments.

FIG. 4. Ouabain on short-circuit current (SCC) of isolated toad bladder. At the arrow, ouabain (8.3×10^{-5}M) was added to media bathing the serosal side of a hemibladder. The other hemibladder served as a simultaneous untreated control. Both bladders were continuously short-circuited, including the 4 hr equilibration period preceding addition of the drug.

tions of 10^{-6} M or lower had no effect on the scc of the isolated toad bladder when added to the media bathing the serosal side of the tissue. Higher concentrations (10^{-5}-10^{-3} M) produced only a slight but transient effect on the scc. Fig. 4 shows the typical transient decrease in scc following addition of ouabain (8.3×10^{-5} M) to the serosal media. Note the recovery of the scc approximately 30 minutes after the drug was added, with a subsequent increase in scc above the pre-drug value.

b. *Effects of aldosterone.* Addition of aldosterone (7×10^{-7} M) to the serosal media was found to produce a sustained increase in the scc compared to the control bladder. In confirmation of the results seen by Crabbe(1) and Edelman and associates (3,4), approximately a 60-90 minute latent period was seen before a clear increase in the scc was noted. Addition of ouabain in a concentration of 8.3×10^{-5} M failed to affect the aldosterone-stimulated sodium transport when the glycoside was added at 340 minutes. These effects are illustrated in Fig. 5.

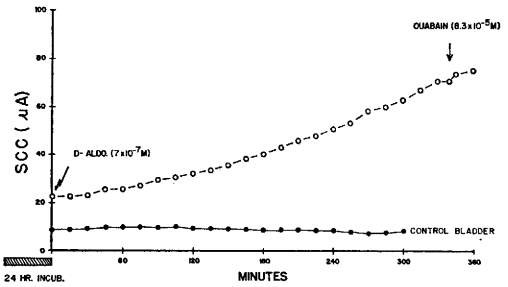

FIG. 5. Aldosterone on short-circuit current (SCC) of isolated toad bladder. Both hemibladders were "incubated" for 24 hr (plus 30 min glucose pretreatment) before aldosterone (7×10^{-7}M) was added to the serosal media of experimental bladder. Ouabain (8.3×10^{-5}M) added to the serosal media of aldosterone stimulated bladder at 340 min failed to change the SCC.

Discussion. a. *Effects of aldosterone.* Agents which increase the scc, and thus active Na^+ transport in the isolated toad bladder may do so by several mechanisms, including (a) action on the transport (carrier) mechanism, (b) action on the metabolic or enzymatic sources supplying energy to the transport mechanism, (c) modification of the access of Na^+ to the carrier. Vasopressin apparently increases active Na^+ transport in the toad bladder predominantly by the latter mechanism, even though the hormone is capable of stimulating the energy metabolism of the tissue(7,8). The stimulation of energy metabolism by vasopressin, including an increased O_2 uptake, was thus found to be entirely secondary to the direct effect on active Na^+ transport as indicated by the lack of effect of the hormone on the metabolism of the tissue in the absence of Na in the incubating medium(7).

On the other hand, thyroxine, which also increases O_2 uptake of both the isolated skin and bladder of the toad concomitant with a stimulation of active Na^+ transport, does so by a mechanism which produces a rise in respiration greatly exceeding that presumably required by the energy-dependent active Na^+ transport mechanism(13). These results have been interpreted as indicating that thyroxine not only stimulates the enzyme systems involved in active Na^+ transport but also those concerned with general cellular metabolism.

While our data show that a low concentration of aldosterone produces a stimulation of O_2 uptake of minced toad bladders from animals kept in a distilled water environment, the question may be raised as to whether this is a non-specific effect on cellular respiration or whether in fact the steroid is stimulating metabolic processes directly involved in the active transport of Na^+ in the intact bladder.

That aldosterone is acting to increase bladder O_2 uptake specifically through an energy-yielding system involved in active Na^+ transport is supported by the following: (a) The greatest effect of aldosterone is seen on minced bladders from toads kept in a water environment known to stimulate the endogenous production of the hormone(2), and which show the greatest sensitivity to the stimulant effects of aldosterone on active Na^+ transport(1). (b) The O_2 uptake response to aldosterone is greater in a glucose-enriched than in a glucose-free media; (c) the increase in O_2 uptake is brought about by a low concentration of steroid shown to be effective in enhancing active Na^+ transport (scc); (d) the stimulation of O_2 uptake by aldosterone in bladders from Group I toads follows a diphasic course, with one rate evident from 0-150 minutes, a second faster rate from 150 minutes on. This may correspond to a similar latent period for increase in Na^+ transport produced by the hormone in the intact bladder(1,3,4).

On the other hand, the results may be interpreted by the following: (a) aldosterone, perhaps like other steroids, may act to uncouple phosphorylation from oxidation(14). However, arguing against this view are reports indicating that uncoupling agents such as 2,4 dinitrophenol decrease the active transport of Na^+ in the toad bladder(15) and frog skin(16) with a concomitant increase in O_2 uptake in the latter. (b) The steroid may have a non-specific stimulating action on cellular respiration unrelated to its action in stimulating the active transport of Na^+. Arguing against this interpretation, however, is the finding that in a wide range of concentrations, aldosterone has no effect on O_2 uptake of rat, rabbit and human myocardium (17).

b. *Effects of ouabain.* In view of the potency of ouabain in inhibiting active Na^+

transport in amphibian tissue such as the frog skin (*e.g.*, 18, 19), as well as in a variety of other tissues, the finding that the scc of the toad bladder was only transiently affected by a high concentration of the glycoside was surprising. Sharp and Leaf(20) and Bower(21) have also indicated that the unstimulated toad bladder scc is little affected by ouabain in concentrations lower than 10^{-4} M. The former could only get inhibition of Na^+ transport in an aldosterone stimulated bladder. We were unable to show a reversal of aldosterone effects with an 8.3×10^{-5} M concentration of ouabain (Fig. 5).

This relative lack of effect of ouabain remains to be explained. One possibility that we are exploring is that the Na^+-K^+ activated ATPase which is glycoside sensitive in other tissues may be relatively unaffected by ouabain in the toad bladder. There is evidence that frog gastric mucosal ATPase does not show the same cation dependence for activation as other membrane ATPase and is not influenced by ouabain up to 10^{-3} M (22).

It is conceivable that bladder membrane ATPase may show a similar pattern of Na^+-K^+ activation which may help explain the relative lack of glycoside effects on active Na^+ transport.

With regard to ouabain effects on bladder O_2 consumption, the data fail to show any significant effect of the glycoside on oxidative metabolism. This lack of a definite effect of ouabain is compatible with its lack of effect on the scc; although it should be emphasized that inhibition of active cation transport by glycosides can occur in the absence of changes in oxidative metabolism(23).

Summary. O_2 consumption of minced urinary bladders of toads which were kept in various environments was measured. A concentration of aldosterone (1.9×10^{-7} M) effective in increasing Na^+ transport was found to produce a significant increase in O_2 uptake in a glucose-enriched media using bladders from toads kept in a distilled water environment. This stimulant effect was less apparent in bladders from toads kept in a moist soil environment. The stimulation of O_2 consumption by aldosterone seen in bladders from toads kept in distilled water was non-linear, with 2 rates being distinguishable, one from 0-150 minutes, the other from 150 minutes on. This change in rate of O_2 consumption may be related to the latent period for stimulation of enzyme synthesis and active Na^+ transport suggested by others. Ouabain was found to produce no significant effect on O_2 consumption of minced bladders. Ouabain (8.3×10^{-5} M) produces only a transient decrease in the short-circuit current of the isolated toad bladder. This is in marked contrast to the potent inhibition produced by the glycoside in frog skin. Although O_2 uptake of tissue in high (18.8 mEq) K^+ media was significantly higher than that seen in a normal (1.88 mEq) solution, steroid effects were absent in the high K^+ environment. The data are compatible with the view that aldosterone produces its effect on active Na^+ transport in part *via* an effect on O_2-dependent energy metabolism.

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Complement-Fixing Activity of Rabbit Antibody Reconstructed from Half Molecules.* (29890)

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The effect of reduction and alkylation of antibody on its complement (C′) fixing properties has been studied in a number of laboratories with conflicting results. After rabbit anti-human γ globulin was reduced and alkylated, we found essentially no decrease in C′-fixing properties(1). Schur and Becker(2) observed a reduction in C′ fixation of less than 20% after treating rabbit anti-human albumin sera with cysteine, whereas Wiedermann *et al*(3,4) recently reported complete loss of the C′-fixing activity associated with human and rabbit antibodies after reduction in 0.1 M mercaptoethanol. As the exact condition for the reduction-alkylation treatment was different in each investigation, it is difficult to decide from these experiments which molecular structures are essential for the C′-fixing properties of antibody. Recently, Palmer *et al*(5) reported that rabbit γ globulin was split into half molecules by reduction in 0.1 M mercaptoethylamine followed by lowering the pH to 2.5, indicating that the inter-heavy (H) chain disulfide linkage(s) is reduced by the procedure. In view of these findings, the present experiments were undertaken to study the role of the inter-H chain disulfide linkages in rabbit antibody molecules on their C′-fixing properties. The results indicate that after irreversible reduction of the inter-H chain disulfide linkages, the half molecules of antibody can recombine in the presence of antigen to form antibody complexes which fix C′.

Materials and methods. Antigen and antibody. A crystalline bovine serum albumin (BSA) (Armour & Co.) rabbit anti-BSA system was employed in these experiments. Anti-BSA rabbit serum was obtained by immunization of rabbits for 4 weeks with an increasing amount of alum-precipitated BSA. The animals were bled 7 days after the last injection of antigen. Gamma globulin fraction of the antiserum was obtained by precipitation with ammonium sulfate at ⅓ saturation followed by DEAE cellulose chromatography(6).

Reduction of γ globulin. The γ globulin fraction of anti-BSA serum was reduced with mercaptoethylamine HCl (Nutritional Biochem. Co.) under N_2 by the method of Palmer *et al*(5). The fraction (1.5%) was incubated at pH 5.0 in 0.1 M mercaptoethylamine for 75 minutes at 37°C. Twice molar excess of iodoacetate was then added to the reaction mixture and the pH maintained at 8.0 by addition of 1 M Tris (Tris (hydroxymethyl) aminoemethane) solution. After one hour, the reduced-alkylated γ globulin was dialyzed against saline for 48 hours.

Complement fixation tests. Pooled guinea pig serum was used as the source of C′. The serum was purchased in a frozen state from Probio Inc., New York, and absorbed with 1/30 volume of packed sheep erythrocytes. The number of units of C′ inactivated by antigen-antibody complexes was determined

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