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FURTHER STUDIES ON THE EFFECT OF ALDOSTERONE ON GLUCOSE METABOLISM IN TOAD BLADDER

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SUMMARY

Aldosterone stimulates Na^+ transport across the toad bladder. Simultaneously, the evolution of $^{14}CO_2$ from [1-^{14}C]- — [6-^{14}C]glucose is decreased. This decrease is due to inhibition of the hexose monophosphate shunt pathway. Because of similarities in the concentration dependence, time course, and other characteristics of the effect of aldosterone on Na^+ transport and on $^{14}CO_2$ evolution from [1-^{14}C]glucose, the possibility exists that these effects are directly related. An attempt has been made to determine whether the effect on the hexose monophosphate shunt pathway is related to the mineralocorticoid effect of aldosterone or to other attributes such as the glucocorticoid capacity of this hormone. It has been shown that spirolactone SC 14266, a structural competitive inhibitor of aldosterone which inhibits the effect of aldosterone on Na^+ transport, inhibits the effects of aldosterone on glucose metabolism. Further, it is shown that aldosterone and deoxycorticosterone can induce the effect on glucose metabolism at low concentrations ($5 \cdot 10^{-9}$ M) whereas at the same concentration cortisol fails to inhibit the hexose monophosphate shunt pathway. Cortisone, which is inactive on Na^+ transport *in vitro*, and cortexolone which is very weakly active, are inactive on glucose metabolism at $1 \cdot 10^{-7}$ M. Thus the stimulation of Na^+ transport and the inhibition of the hexose monophosphate shunt pathway both appear to be effects of mineralocorticoids and the latter does not represent a separable glucocorticoid effect.

INTRODUCTION

Aldosterone has been shown to have several effects upon the toad bladder. It stimulates Na^+ transport[1–3], oxygen consumption, and substrate metabolism[4–6]. Stimulation of metabolism is thought to be directly related to the greater work load associated with the increased rate of Na^+ transport. In the absence of Na^+, metabolism is not enhanced[4,6]. One metabolic effect of aldosterone, which is not Na^+ dependent, is the inhibition of $^{14}CO_2$ release from [1-^{14}C]glucose. In the interpretation of experiments using ^{14}C-labeled glucose, we have assumed that $^{14}CO_2$ evolution from [6-^{14}C]-

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glucose reflects the utilization of glucose *via* th Embden–Meyerhof pathway. $^{14}CO_2$ evolution from [1-^{14}C]glucose reflects the utilization of glucose from the Embden–Meyerhof pathway *plus* alternate pathways, the principal one of which is the hexose monophosphate shunt pathway. As the C-1 and C-6 atoms of glucose are treated similarly by the Embden–Meyerhof pathway, the amount of $^{14}CO_2$ from [6-^{14}C]-glucose can be subtracted from that of [1-^{14}C]glucose to represent $^{14}CO_2$ from the alternate pathways. This remaining CO_2 evolution may, in large part, be ascribed to the hexose monophosphate shunt pathway and is reduced by aldosterone. The large magnitude of this effect has led to the assumption that this is due to inhibition of the hexose monophosphate shunt pathway. It was thought worthwhile, however, to document the inhibition by additional studies. Thus pathway separation by the method of WOOD *et al.*[8] utilizing ^{14}C labeling of a triose derivative by [1-^{14}C]- and [6-^{14}C]glucose was performed. Also information on the extent of recycling was obtained by the use of [2-^{14}C]glucose.

The primary nature of the effect led us to test the relationship between this effect of aldosterone on glucose metabolism and the effect of aldosterone on Na^+ transport[7]. It was shown that both effects had the same time course of onset and concentration dependence. Both effects were abolished by actinomycin D and exhibited steroid specificity. Aldosterone, deoxycorticosterone, cortisol, and dexamethasone, all of which stimulate Na^+ transport, have the ability to inhibit the hexose monophosphate shunt pathway whereas progesterone, which is inactive on Na^+ transport, was not inhibitory[7]. It was concluded that either the effects on glucose metabolism and Na^+ transport were related or that the effect on glucose metabolism was a coincidental steroid effect, perhaps a "glucocorticoid" effect, of aldosterone.

The following experiments were performed in an attempt to distinguish between these two possibilities. In the first series of experiments, a study was made of the ability of spirolactone SC 14266 to antagonize the effects of aldosterone and of dexamethasone on glucose metabolism. Since spirolactone SC 14266 is known to block the "mineralocorticoid" effect of aldosterone on Na^+ transport, it was of interest to ascertain if spirolactone SC 14266 simultaneously blocks the effect on [1-^{14}C]-glucose metabolism. In the second series of experiments, a comparison was made of the effects on glucose metabolism of aldosterone, cortisol, and deoxycorticosterone (with its high "mineralocorticoid" activity relative to "glucocorticoid" activity). It was hoped that by using low concentrations of these steroids, an estimate of the relative potency of mineralocorticoids *versus* glucocorticoids could be obtained for the effect on glucose metabolism. In addition, the effects of cortisone and cortexolone were studied. Cortisone is inactive on Na^+ transport[3] and cortexolone only weakly active[9].

METHODS

All experiments were performed on bladders of the toad, *Bufo marinus* (obtained from National Reagents, Bridgeport, Conn.). Toads were maintained partially immersed in 0.6 % saline solutions for 2–5 days prior to use to decrease the endogenous secretion of aldosterone, and then rapidly pithed.

(*a*) *Pathway separation*

Half-bladders were incubated in 5 ml of frog Ringer's solution (NaCl, 113.5; KCl, 2.5; $NaHCO_3$, 2.4; and $CaCl_2$, 0.89 mM; total solute concentration, 220 mosM/kg water; pH in air 7.8). Penicillin G and streptomycin (0.1 mg/ml) each were included in all incubation media used in this study. Half-bladders were incubated in Ringer's solution in a rotatory metabolic shaker with or without $1 \cdot 10^{-7}$ M (+)-aldosterone. At the end of 5.5 h (or 6 and 16 h in two experiments) the half-bladders were washed in Ringer's solution and reincubated in fresh Ringer's solution for a further 15 min. The wash procedure was repeated in order to ensure the removal of excess lactate from the solution and tissue. A final wash was performed before the bladders were quartered and incubated for 2 h as follows. One quarter-bladder which had been treated with aldosterone was incubated in 5 ml Ringer's solution containing 5.5 mM glucose and 1 μC of [1-^{14}C]glucose*; the other quarter-bladder in 5 ml Ringer's solution containing 5.5 mM glucose and 1 μC of [6-^{14}C]glucose. The two control quarter-bladders were incubated in similar solutions. At the end of the incubation period, the quarter-bladders were removed from the flasks and discarded. CO_2 was driven off from the 5-ml medium by the addition of 0.2 ml of 0.5 M H_2SO_4. Lactate was isolated by the methods of BUSCH *et al.*[10] as follows: The medium was titrated with 1 M KOH until just alkaline using phenol red as the indicator and placed on a 15-cm Dowex 1-X8, 200–400 mesh, anion-exchange column in formate form. [^{14}C]-Glucose was eluted from the column with 30 ml of water made alkaline with 1 drop of 1 M KOH and discarded. Lactic acid was separated by eluting with a 0–6 M formic acid gradient. 1.5–2.0 ml fractions were collected and lactate was identified by comparison with isotopically labeled lactate separated under the same conditions. The isolated lactate in formic acid solution was evaporated to dryness under vacuum at at emperature not exceeding 40°. The lactate was redissolved in water and evaporated to dryness again to ensure complete volatilization of formic acid. The residue was redissolved in water and assayed for lactate by the method of BARKER AND SUMMERSON[11]. During the chemical assay of lactate a final purification step, treatment with $CuSO_4$ and $Ca(OH)_2$, was introduced. The supernatant from this step was used for the colorimetric assay of lactate. To determine the amount of ^{14}C-labeled lactate present, a 500-μl aliquot was counted in 15 ml dioxane scintillation fluid in a Nuclear Chicago Mark I scintillation spectrometer. Medium samples were counted similarly. From the results the specific activity of labeled lactate derived from [1-^{14}C]glucose and [6-^{14}C]glucose was determined.

(*b*) *To determine the effect of aldosterone upon $^{14}CO_2$ release from [1-^{14}C]-, [2-^{14}C]-, and [6-^{14}C]glucose*

To determine this effect bladders were divided into 1/6 portions and incubated at room temperature for 6 h in Ringer's solution. Three pieces of bladder were incubated with aldosterone ($5 \cdot 10^{-7}$ M). The remaining three served as control tissues. Measurements of glucose utilization were started by dropping the tissues into flasks of fresh Ringer's solution containing 5.5 mM glucose and radioactively labeled glucose. One of each of the three pieces was incubated in such a flask to which [1-^{14}C]glucose had

* [^{14}C]Glucose was obtained from New England Nuclear Corporation, Boston, Mass. Purity was greater than 99% and was checked by thin-layer silica-gel chromatography in an ethyl acetate–isopropanol–water solvent system (130:75:30, by vol.) followed by radioautography.

been added, one to which [2-^{14}C]glucose had been added, and the other to a flask with [6-^{14}C]glucose. The flasks were sealed and incubated at room temperature for 60 min. At the end of this time 0.2 ml of 1.0 M H_2SO_4 was injected into the medium of each flask and the flasks shaken for an additional 30 min. The CO_2 liberated was trapped in a center well with 25 % KOH on a filter paper wick.

The filter paper wick and the contents of the center well were quantitatively transferred, with 3.0 ml of methanol, to vials for liquid scintillation counting and 10 ml of a toluene scintillation mixture added. Samples of the medium were also taken for radioactive counting. The tissues were removed, blotted lightly, transferred to tared weighing tubes, and dry weights obtained. Glucose utilization was calculated as μmoles/g dry weight per h. Similar incubations were performed simultaneously but without tissue in order to obtain blanks for the radioactivity.

(c) To determine the effect of spirolactone SC 14266 upon $^{14}CO_2$ release from [1-^{14}C]- and [6-^{14}C]glucose

Three pairs of 1/8 bladders were incubated for 6 h in Ringer's solution containing three different concentrations of spirolactone SC 14266 (10^{-4} M, 10^{-5} M and 10^{-6} M). The remaining pair served as control tissue. Glucose utilization was estimated as described in (*b*), one of each pair being incubated with [1-^{14}C]glucose and the other with [6-^{14}C]glucose.

(d) To determine the effects of spirolactone SC 14266 and aldosterone upon $^{14}CO_2$ release from [1-^{14}C]- and [6-^{14}C]glucose

Pairs of 1/8 portions of bladders were incubated at room temperature for 6 h in Ringer's solution containing the following combinations of aldosterone and spirolactone. (1) (+)-Aldosterone; (2) spirolactone SC 14266; (3) (+)-aldosterone + spirolactone SC 14266; (4) no additions. Glucose utilization was measured as described in (*b*). Two series of experiments were performed with aldosterone at a concentration of $2.5 \cdot 10^{-9}$ M. In the first, a spirolactone concentration of $2.5 \cdot 10^{-6}$ M was used and in the second, $5.0 \cdot 10^{-6}$ M.

(e) To determine the effects of spirolactone SC 14266 and dexamethasone upon $^{14}CO_2$ release from [1-^{14}C]-and [6-^{14}C]glucose

One series of experiments was performed as in (*b*) using spirolactone SC 14266 at $5 \cdot 10^{-6}$ M and dexamethasone at $1 \cdot 10^{-8}$ M. A second series of experiments was performed using spirolactone SC 14266 at $5 \cdot 10^{-6}$ M and dexamethasone at $2.5 \cdot 10^{-9}$ M, a competition ratio of 2000:1. In this series, only tissues which showed an increase in evolution of $^{14}CO_2$ from [6-^{14}C]glucose and a decrease from [1-^{14}C]- and [6-^{14}C]-glucose with dexamethasone alone were examined for the effect of spirolactone SC 14266 and dexamethasone combined.

(f) To determine the relative potencies of aldosterone, deoxycorticosterone, and cortisol with respect to their effects upon glucose metabolism

Pairs of 1/8 portions of bladder were incubated at room temperature for 6 h in Ringer's solution containing either: (1) Aldosterone, (2) deoxycorticosterone, (3) cortisol, (4) methanol diluent as controls. The concentration of each steroid was $5 \cdot 10^{-9}$ M. Glucose utilization was measured as described in (*b*).

(g) To determine the effects of cortisone and cortexolone upon $^{14}CO_2$ release from [1-^{14}C]- and [6-^{14}C]glucose

The determinations were carried out as described above after incubating paired tissues with the steroid at $1 \cdot 10^{-7}$ M and methanol diluent.

RESULTS

(a) Pathway separation

WOOD *et al.*[8] have devised methods for pathway separations based on the following formulation of the pentose cycle.

3 glucose-6-*P* → 3 CO_2 + 3 pentose-*P*

3 pentose-*P* → 2 fructose-6-*P* + 1 glyceraldehyde-3-*P*

2 fructose-6-*P* → 2 glucose-6-*P*

∴ 1 glucose-6-*P* → 3 CO_2 + 1 glyceraldehyde-3-*P*

This model assumes complete recycling of fructose-6-*P* to glucose-6-*P*. This recycling will dilute the specific activity of the labeled glucose-6-*P* when [1-^{14}C]-glucose, but not [6-^{14}C]glucose, is the substrate because of the loss of $^{14}CO_2$ *via* the pentose cycle. When the hexose monophosphate shunt (HMP) pathway and the Embden–Meyerhof (EM) pathway are the sole pathways of glucose metabolism, it can be shown that the dilution factor, called $Q = 1/(1 + 2\text{HMP})$.

As the trioses and derivatives (such as lactate) are labeled by [1-^{14}C]glucose only *via* the EM pathway and by [6-^{14}C]glucose *via* both the EM and HMP pathways, then the ratio of the triose labeling may be derived as follows.

$$\frac{1\text{-}^{14}C}{6\text{-}^{14}C} = \frac{EM \times Q}{EM + HMP}$$

but $EM = 1 - HMP$

$$\therefore \frac{1\text{-}^{14}C}{6\text{-}^{14}C} = \frac{1 - HMP \cdot Q}{1}$$

substituting $1/(1 + 2\ HMP)$ for Q

$$\frac{1\text{-}^{14}C \text{ in triose}}{6\text{-}^{14}C \text{ in triose}} = \frac{1 - HMP}{1 + 2HMP}$$

Hence, the extent of the HMP can be determined. The assumptions underlying this method have been described in detail[8]. The results of four unpaired determinations are shown in Table I and seven paired determinations in Table II. In all cases, the pentose cycle was reduced in tissues treated with aldosterone. The mean values for the percent contribution of the pentose cycle to total glucose metabolism for paired determinations were: control tissues, 22.5 %; aldosterone-treated tissues, 7.8 %. The reduction of 14.6 ± 2.9 % was highly significant ($P < 0.01$)

TABLE I

DATA AND CALCULATION OF PENTOSE CYCLE CONTRIBUTION (%) TO GLUCOSE METABOLISM IN 4 UNPAIRED CONTROL AND ALDOSTERONE-TREATED ($5 \cdot 10^{-7}$ M) TOAD BLADDER ACCORDING TO A METHOD OF WOOD *et al.*[8]

The specific activity correction factor is required because of differences in the specific activity of glucose in the media.

Incubation time (h)	*Treatment*	*Label position*	*Glucose counts/min per 50 µl medium*	*Counts/min per ml lactate sample*	*Lactate in sample (counts/min per µg)*	*Spec. act. lactate (counts/min per µg)*	*Spec. act. correction factor*	*Corrected spec. act.*	$\frac{\text{C-1 spec. act.}}{\text{C-6 spec. act.}}$	*Pentose cycle* (%)
16	Control	C-1	8360	525	4.76	110	0.904	99	0.655	15
		C-6	7310	628	4.16	151	—	151		
16	Aldosterone	C-1	4199	104	7.42	14.0	0.974	13.6	0.919	2.8
		C-6	4094	132	8.92	14.8	—	14.8		
6	Control	C-1	16 986	332	2.32	143	0.904	129	0.408	32.3
		C-6	15 367	284	0.90	316	—	316		
6	Aldosterone	C-1	33 916	666	3.50	190	0.976	185	0.794	8.0
		C-6	33 092	560	2.40	233	—	233		

TABLE II

DATA AND CALCULATION OF PENTOSE CYCLE CONTRIBUTION (%) TO GLUCOSE METABOLISM IN 7 PAIRED CONTROL AND ALDOSTERONE-TREATED ($5 \cdot 10^{-7}$ M) TOAD BLADDERS ACCORDING TO A METHOD OF WOOD *et al.*[8]

Expt. No.	*Incubation time (h)*	*Treatment*	*Label position*	*Glucose counts/min per 50 µl medium*	*Counts/min per ml lactate solution*	*Lactate (µg/ml)*	*Spec. act. lactate (counts/min per µg)*	*Spec. act. correction factor*	*Corrected spec. act.*	*C-1 spec. act. / C-6 spec. act.*	*Pentose cycle (%)*
1	6	Control	C-1	49 020	145	1.24	117	0.845	99	0.589	18.4
			C-6	41 398	151	0.90	168	—	168		
		Aldosterone	C-1	51 404	136	0.80	170	0.824	140	0.725	11.2
			C-6	42 332	139	0.72	193	—	193		
2	6	Control	C-1	16 963	961	3.78	254	0.901	229	0.443	29.5
			C-6	15 292	652	1.26	517	—	517		
		Aldosterone	C-1	17 271	1286	10.98	117	0.883	103	0.613	17.4
			C-6	15 427	1052	6.30	167	—	168		
3	5	Control	C-1	33 752	657	3.40	193	0.972	188	0.644	15.6
			C-6	32 790	847	2.90	292	—	292		
		Aldosterone	C-1	33 950	580	2.96	196	0.971	190	0.826	6.6
			C-6	32 978	764	3.32	230	—	230		
4	5	Control	C-1	33 886	291	2.96	98	0.975	96	0.527	23.0
			C-6	33 035	639	3.52	181	—	182		
		Aldosterone	C-1	33 977	460	2.78	165	0.968	160	0.773	8.9
			C-6	32 888	755	3.64	207	—	207		
5	6	Control	C-1	36 913	912	1.72	530	—	530	0.693	12.9
			C-6	37 436	1552	2.00	776	0.986	765		
		Aldosterone	C-1	37 458	1294	1.70	761	0.995	757	1.070	0.0
			C-6	37 350	1310	1.86	704	—	704		
6	6	Control	C-1	39 578	412	1.82	226	0.930	210	0.405	32.9
			C-6	31 721	1381	2.66	519	—	519		
		Aldosterone	C-1	37 439	456	1.72	265	0.837	222	0.941	2.4
			C-6	31 344	627	2.66	236	—	236		
7	6	Control	C-1	38 849	826	2.30	359	0.901	323	0.498	25.1
			C-6	35 000	1323	2.04	648	—	648		
		Aldosterone	C-1	38 400	1025	3.56	288	0.910	262	0.784	8.4
			C-6	34 928	991	2.97	334	—	334		

(b) The effect of aldosterone upon $^{14}CO_2$ release from [1-^{14}C]-, [2-^{14}C]- and [6-^{14}C]glucose

The pathway separation described in (*a*) assumes complete recycling. If recycling were not complete, then one means of decreasing $^{14}CO_2$ from [1-^{14}C] — [6-^{14}C]-glucose would be to increase the extent of recycling. This has the effect of decreasing the specific activity of the glucose 6-phosphate pool by removing the ^{14}C from [1-^{14}C]glucose and returning unlabeled glucose 6-phosphate *via* fructose 6-phosphate. As an increase in recycling can be detected by a decrease in $^{14}CO_2$ from [1-^{14}C] — [6-^{14}C]glucose and by an increase in $^{14}CO_2$ from [2-^{14}C] — [6-^{14}C]glucose, the effect of aldosterone on the evolution of $^{14}CO_2$ from [1-^{14}C]-, [2-^{14}C]- and [6-^{14}C]glucose was measured. The results are shown in Table III.

TABLE III

EFFECTS OF ALDOSTERONE ($5 \cdot 10^{-7}$ M) ON THE METABOLISM OF [1-^{14}C]-, [2-^{14}C]- AND [6-^{14}C]GLUCOSE IN TOAD BLADDER

Results are expressed as μmoles of glucose utilized per g dry weight of tissue per h.

	Control	*Aldosterone*	$\Delta \pm$ S.E.	*Change* (%)	*n*	*P*
C-1	1.92	1.68	-0.24 ± 0.21	—	10	0.3
C-2	1.02	1.15	0.13 ± 0.17	—	10	0.5
C-6	0.67	1.13	0.46 ± 0.13	69	10	<0.01
C-1—C-6	1.25	0.55	-0.70 ± 0.17	−56	10	<0.01
C-2—C-6	0.35	0.03	-0.32 ± 0.11	−91	10	<0.02

That recycling occurs in both control and aldosterone-treated tissues is shown by the fact that $^{14}CO_2$ evolution from [2-^{14}C]glucose is greater than that from [6-^{14}C]-glucose under both conditions. $^{14}CO_2$ from [2-^{14}C]glucose is released in the hexose-monophosphate shunt pathway only after the molecule has passed down the pathway, has the unlabeled carbon removed from the 1 position and recycles to glucose 6-phosphate so that the original 2-^{14}C is now in the 1 position. This radioactive carbon is removed during the second passage through the cycle (*i.e.* after recycling). The effect of aldosterone to decrease $^{14}CO_2$ from [1-^{14}C] — [6-^{14}C]glucose is seen clearly. More importantly, the $^{14}CO_2$ from [2-^{14}C] — [6-^{14}C]glucose is also significantly decreased. Thus recycling cannot be increased by aldosterone and inhibition of the hexosemonophosphate shunt pathway must have occurred.

(c) The effect of spirolactone SC 14266 upon $^{14}CO_2$ release from [1-^{14}C]- and [6-^{14}C]-glucose

Before studying the antagonism of spirolactone SC 14266 and aldosterone it was necessary to ensure that the spirolactone SC 14266 did not have any effect upon glucose metabolism itself. Table IV shows the effect of spirolactone SC 14266 at concentrations of 10^{-4} M, 10^{-5} M, and 10^{-6} M.

At 10^{-4} M, spirolactone SC 14266 significantly decreased the $^{14}CO_2$ evolution from [1-^{14}C]glucose and also decreased, though not significantly, the $^{14}CO_2$ evolution from [6-^{14}C]glucose. At this concentration an immediate and nonspecific inhibition of sodium transport is also observed[12]. At 10^{-5} M spirolactone SC 14266 a non-significant decrease of $^{14}CO_2$ evolution occurred while at 10^{-6} M no effect of spiro-

TABLE IV

EFFECTS OF SPIROLACTONE SC 14266, AT THREE DIFFERENT CONCENTRATIONS, ON THE METABOLISM OF [1-^{14}C]- AND [6-^{14}C]GLUCOSE BY TOAD BLADDER

Results are expressed as μmoles of glucose utilized per g dry weight of tissue per h.

	Control	*SC 14266* 10^{-4} *M*	$\Delta \pm$ *S.E.*	*n*	*P*
C-1	1.85	1.36	-0.49 ± 0.11	7	<0.01
C-6	0.80	0.62	-0.18 ± 0.10	7	<0.20
C-1 — C-6	1.05	0.74	-0.31 ± 0.11	7	<0.05
	Control	*SC 14266* 10^{-5} *M*	$\Delta \pm$ *S.E.*	*n*	*P*
C-1	1.85	1.60	-0.25 ± 0.18	7	<0.20
C-6	0.80	0.77	-0.03 ± 0.10	7	0.80
C-1 — C-6	1.05	0.83	-0.22 ± 0.15	7	<0.20
	Control	*SC 14266* 10^{-6} *M*	$\Delta \pm$ *S.E.*	*n*	*P*
C-1	1.85	1.90	$+0.05 \pm 0.11$	7	0.6
C-6	0.80	0.81	$+0.01 \pm 0.07$	7	0.9
C-1 — C-6	1.05	1.10	$+0.05 \pm 0.10$	7	0.7

lactone SC 14266 upon glucose metabolism was detectable. Consequently, concentrations of spirolactone SC 14266 between 10^{-6} and 10^{-5} M were used for the antagonism studies. These concentrations, however, restrict the concentration of aldosterone which may be used because of the need to maintain a spirolactone excess of at least 1000-fold that of the hormone.

(d) The effects of spirolactone SC 14266 ($2.5 \cdot 10^{-6}$ M and $5.0 \cdot 10^{-6}$ M) and aldosterone ($2.5 \cdot 10^{-9}$ M) upon $^{14}CO_2$ release from [1-^{14}C]- and [6-^{14}C]glucose

It can be seen in Table V that spirolactone SC 14266 at $2.5 \cdot 10^{-6}$ M had no effect upon the $^{14}CO_2$ evolution from either [1-^{14}C]- or [6-^{14}C]glucose. It is also clear that aldosterone produced its typical effects with an 83% increase in the $^{14}CO_2$ from [6-^{14}C]glucose and a 46% decrease in the [1-^{14}C] — [6-^{14}C]parameter. When the tissue was subjected to both aldosterone and spirolactone, the effect of the hormone was inhibited and no longer significantly different from control values. Thus, a 1000-fold excess of spirolactone SC 14266 inhibits, though not completely, both the increase in $^{14}CO_2$ from [6-^{14}C]glucose and the decrease in the [1-^{14}C] — [6-^{14}C]parameter. In the second series of experiments performed with a 2000-fold excess of spirolactone SC 14266 (see Fig. 1), inhibition of the effect of aldosterone upon [1-^{14}C] — [6-^{14}C]glucose utilization was almost complete. Spirolactone SC 14266 at these concentrations had no effect in the absence of aldosterone.

(e) The effects of spirolactone SC 14266 ($5 \cdot 10^{-6}$ M) and dexamethasone ($1 \cdot 10^{-8}$ M) upon $^{14}CO_2$ release from [1-^{14}C]- and [6-^{14}C]glucose

The results in Table VI show that dexamethasone at $1 \cdot 10^{-8}$ M causes a significant increase in $^{14}CO_2$ from [6-^{14}C]glucose and a significant decrease in $^{14}CO_2$ from [1-^{14}C]

TABLE V

EFFECTS OF ALDOSTERONE ($2.5 \cdot 10^{-9}$ M) AND SPIROLACTONE SC 14266 ($2.5 \cdot 10^{-6}$ M) ON THE METABOLISM OF [1-^{14}C]- AND [6-^{14}C]GLUCOSE BY TOAD BLADDER

Results are expressed as μmoles of glucose utilized per g dry weight of tissue per h.

	Control	*Aldosterone*	$\Delta \pm$ S.E.	*Change* (%)	*n*	*P*
C-1	1.92	2.03	$+0.11 \pm 0.18$		11	0.6
C-6	0.84	1.54	$+0.70 \pm 0.12$	+83	11	<0.01
C-1 — C-6	1.08	0.49	-0.59 ± 0.16	−55	11	<0.01
	Control	*SC 14266*	$\Delta \pm$ S.E.	*Change* (%)	*n*	*P*
C-1	1.92	1.82	-0.10 ± 0.20		11	0.6
C-6	0.84	0.76	-0.08 ± 0.09	−10	11	0.5
C-1 — C-6	1.08	1.06	-0.02 ± 0.22	− 2	11	0.9
	Control	*Aldosterone + SC 14266*	$\Delta \pm$ S.E.	*Change* (%)	*n*	*P*
C-1	1.92	1.85	-0.07 ± 0.18		11	0.7
C-6	0.84	1.02	$+0.18 \pm 0.09$	+21	11	<0.1
C-1 — C-6	1.08	0.83	-0.25 ± 0.15	−23	11	<0.2

— [6-^{14}C]glucose. Spirolactone SC 12466 alone was without effect but clearly decreased the effect of dexamethasone both on the increase of $^{14}CO_2$ from [6-^{14}C]-glucose and on the decrease from [1-^{14}C] — [6-^{14}C]glucose. That this inhibition by spirolactone is only partial at this concentration is shown by the fact that the tissues treated with dexamethasone and spirolactone still maintained a significant increase in $^{14}CO_2$ from [6-^{14}C]glucose and a significant decrease in $^{14}CO_2$ from [1-^{14}C] —

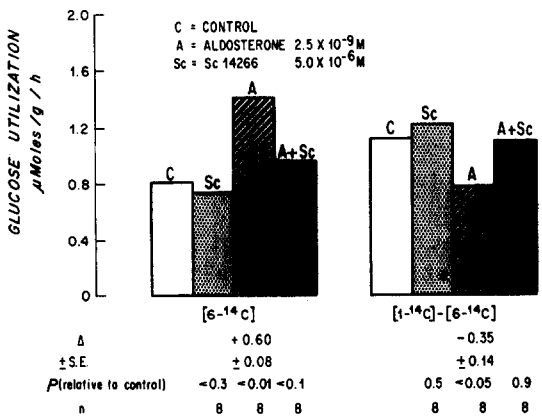

Fig. 1. Effects of aldosterone ($2.5 \cdot 10^{-9}$ M) and spirolactone SC 14266 ($5.0 \cdot 10^{-6}$ M), on the metabolism of [1-^{14}C]- and [6-^{14}C]glucose by toad bladder. Results are expressed as μmoles of glucose utilized per g dry weight of tissue per h.

TABLE VI

EFFECTS OF DEXAMETHASONE ($1 \cdot 10^{-8}$ M) AND SPIROLACTONE SC 14266 ($5 \cdot 10^{-6}$ M) ON THE METABOLISM OF [1-^{14}C]- AND [6-^{14}C]GLUCOSE BY TOAD BLADDER

Results are expressed as μmoles of glucose utilized per g dry weight of tissue per h.

	Control	*Dexamethasone*	$\Delta \pm$ S.E.	*Change* (%)	*n*	*P*
C-1	1.60	1.60	0.0 ± 0.14		11	0.9
C-6	0.61	1.32	0.71 ± 0.12	+116	11	<0.001
C-1 – C-6	0.99	0.28	-0.71 ± 0.12	− 72	11	<0.001
	Control	*SC 14266*	$\Delta \pm$ S.E.	*Change* (%)	*n*	*P*
C-1	1.60	1.49	-0.11 ± 0.18		11	0.6
C-6	0.61	0.56	-0.05 ± 0.07	− 8	11	0.6
C-1 – C-6	0.99	0.93	-0.06 ± 0.15	− 6	11	0.6
	Control	*Dexamethasone + SC 14266*	$\Delta \pm$ S.E.	*Change* (%)	*n*	*P*
C-1	1.60	1.67	0.07 ± 0.12		11	0.6
C-6	0.61	0.96	0.35 ± 0.10	+ 57	11	<0.01
C-1 – C-6	0.99	0.71	0.28 ± 0.10	− 28	11	<0.02

TABLE VII

EFFECTS OF DEXAMETHASONE ($2.5 \cdot 10^{-9}$ M) AND SPIROLACTONE SC 14266 ($5 \cdot 10^{-6}$ M) ON THE METABOLISM OF [1-^{14}C]- AND [6-^{14}C]GLUCOSE BY TOAD BLADDER

Results are expressed as μmoles of glucose utilized per g dry weight of tissue per h.

	Control	*Dexamethasone*	$\Delta \pm$ S.E.	*Change* (%)	*n*	*P*
C-1	1.35	1.25	-0.10 ± 0.08		7	0.3
C-6	0.41	0.65	0.24 ± 0.02	+59	7	<0.001
C-1 – C-6	0.94	0.60	-0.34 ± 0.08	−36	7	<0.01
	Control	*SC 14266*	$\Delta \pm$ S.E.		*n*	*P*
C-1	1.35	1.27	-0.08 ± 0.12		7	0.5
C-6	0.41	0.43	$+0.02 \pm 0.06$		7	0.8
C-1 – C-6	0.94	0.84	-0.10 ± 0.13		7	0.5
	Control	*Dexamethasone + SC 14266*	$\Delta \pm$ S.E.		*n*	*P*
C-1	1.35	1.25	-0.10 ± 0.12		7	0.5
C-6	0.41	0.51	$+0.10 \pm 0.09$		7	0.4
C-1 – C-6	0.94	0.74	-0.20 ± 0.12		7	0.2

[6-^{14}C]glucose. For this reason the spirolactone to dexamethasone ratio was raised from 500:1 to 2000:1 by decreasing the concentration of dexamethasone used.

Under these conditions, it is clear that in tissues which responded to dexamethasone by an increased evolution of $^{14}CO_2$ from [6-^{14}C]glucose and a decreased evolution of $^{14}CO_2$ from [1-^{14}C]glucose, the effect of spirolactone SC 14266 was to prevent the normal action of dexamethasone on both parameters (see Table VII); tissues treated with both dexamethasone and spirolactone were not significantly different from control tissues.

(f) Relative potencies of aldosterone, deoxycorticosterone, and cortisol with respect to their effects on glucose oxidation

Fig. 2 shows the effects of these steroids at the same low concentration ($5.0 \cdot 10^{-9}$ M) on the evolution of $^{14}CO_2$ from [1-^{14}C]- and [6-^{14}C]glucose by paired tissues.

$^{14}CO_2$ evolution from [6-^{14}C]glucose was significantly increased by aldosterone and deoxycorticosterone but not by cortisol. Similarly the $^{14}CO_2$ evolution from [1-^{14}C] — [6-^{14}C]glucose was significantly decreased by aldosterone and deoxycorticosterone but not by cortisol. It appears, therefore, with respect to the inhibition of $^{14}CO_2$ release from [1-^{14}C] — [6-^{14}C]glucose that the two potent mineralocorticoids aldosterone and deoxycorticosterone are more effective than cortisol.

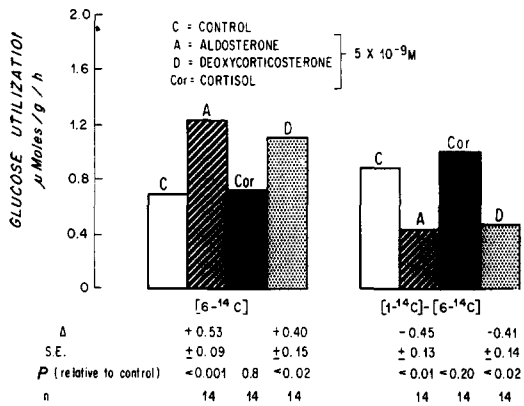

Fig. 2. Effects of aldosterone ($5 \cdot 10^{-9}$ M), deoxycorticosterone ($5 \cdot 10^{-9}$ M) and cortisol ($5 \cdot 10^{-9}$ M) on the metabolism of [1-^{14}C]- and [6-^{14}C]glucose by toad bladder. Results are expressed as μmoles of glucose utilized per g dry weight of tissue per h.

(g) The effects of cortisone ($1 \cdot 10^{-7}$ M) and cortexolone ($1 \cdot 10^{-7}$ M) on $^{14}CO_2$ release from [1-^{14}C]- and [6-^{14}C]glucose

Cortisone and cortexolone had no significant effect upon glucose metabolism at this concentration (Table VIII).

DISCUSSION

The activity of the hexosemonophosphate shunt pathway has been determined in toad bladder, under control conditions and after treatment with aldosterone. Previous results have shown that the evolution of $^{14}CO_2$ from [1-^{14}C] — [6-^{14}C]-

TABLE VIII

EFFECTS OF CORTISONE ($1 \cdot 10^{-7}$ M) AND CORTEXOLONE ($1 \cdot 10^{-7}$ M) ON THE METABOLISM OF [1-^{14}C]- AND [6-^{14}C]GLUCOSE BY TOAD BLADDER

Results are expressed as μmoles of glucose utilized per g dry weight of tissue per h.

	Control	*Cortisone*	$\Delta \pm$ S.E.	*Change* (%)	*n*	*P*
C-1	1.80	1.97	$+0.17 \pm 0.08$		6	<0.1
C-6	0.68	0.71	$+0.03 \pm 0.14$	+ 4	6	0.9
C-1 − C-6	1.12	1.26	$+0.14 \pm 0.10$	+12	6	0.4
	Control	*Cortexolone*	$\Delta \pm$ S.E.	*Change* (%)	*n*	*P*
C-1	2.67	2.43	-0.24 ± 0.28		10	0.4
C-6	0.52	0.64	$+0.12 \pm 0.03$	+23	10	<0.01
C-1 − C-6	2.15	1.79	-0.36 ± 0.27	+17	10	0.2

glucose was markedly decreased by aldosterone. Although it is not possible to estimate the relative pathway activities from $^{14}CO_2$ evolution studies, the magnitude of the decrease due to aldosterone made it extremely likely that the effect was to inhibit the hexosemonophosphate shunt. Consequently, the activities of both the pentose cycle and Embden–Meyerhof pathway were estimated by one of the methods of WOOD *et al.*[8]. The method is based upon the assumptions that the hexose monophosphate shunt and Embden–Meyerhof pathways are the only pathways of glucose metabolism, that recycling of ribose 5-phosphate is complete and that full equilibration occurs between fructose 6-phosphate and glucose 6-phosphate. Aldosterone was found to reduce the hexose monophosphate shunt pathway from 22 to 8 %, a change of large magnitude as has been suggested by the $^{14}CO_2$ evolution studies. Because of this, the possibility that some other pathway of glucose metabolism is involved appears to be extremely unlikely. It is possible, however, that an increase in recycling from a state of little recycling to full recycling could produce such a result. In this case, the reduction of specific activity in glucose 6-phosphate would appear by both $^{14}CO_2$ evolution studies and the method of WOOD *et al.*[8] to reflect an inhibition of the hexose monophosphate shunt pathway. Consequently, studies were performed with [2-^{14}C]-glucose to detect recycling. The ^{14}C from [2-^{14}C]glucose is only released by the hexose-monophosphate shunt pathway during its second passage (*i.e.* after recycling). Thus an increase of $^{14}CO_2$ from [2-^{14}C]- and [6-^{14}C]glucose would reflect increased recycling.

As a decrease was in fact observed, recycling is not contributing to the decrease of $^{14}CO_2$ from [1-^{14}C] — [6-^{14}C]-, or [2-^{14}C] — [6-^{14}C]glucose. The method of WOOD *et al.*[8] for determination of glucose pathways by specific activity of triose derivatives, therefore, indicates that aldosterone does inhibit the hexose mono-phosphate shunt pathway. Thus, the use of $^{14}CO_2$ from [1-^{14}C] — [6-^{14}C]glucose as a convenient and simple indicator of the effect of aldosterone on the hexosemono-phosphate shunt pathway of toad bladder is validated.

In order to study the relationship between the action of aldosterone to stimulate Na^+ transport and its action to inhibit the hexosemonophosphate shunt pathway

in toad bladder, two types of tests for possible dissociation of the effects on Na^+ transport and glucose metabolism have been applied: (1) To determine if the spirolactones, which inhibit the action of aldosterone on Na^+ transport, also inhibit the effect on glucose metabolism. (2a) To compare the potency of the "mineralocorticoid" hormones aldosterone and deoxycorticosterone with that of a "glucocorticoid" hormone cortisol with respect to the glucose effect. (2b) To determine whether steroids that are inactive (or only weakly so) on Na^+ transport had any effect upon glucose metabolism.

The first test applied with the spirolactone had as its basis the work of KAGAWA *et al.*[13] who demonstrated the ability of these compounds to inhibit the Na^+-retaining effects of aldosterone, their lack of effect in the absence of aldosterone, and the similarity of their chemical structures. In toad bladder, the stimulation of Na^+ transport can be inhibited by spirolactones[3,12] and displacement of (+)- [^{3}H]aldosterone from its binding sites in the tissue is evidence that the spirolactones and aldosterone do compete for receptors[14]. More recently, with the demonstration of nuclear receptors for aldosterone, it has been shown that spirolactones displace (+)-[^{3}H]aldosterone from its nuclear sites[15–18]. Complete inhibition by spirolactones of the Na^+ transport response to aldosterone in toad bladder has been demonstrated[6], and PORTER[12], in a detailed study of the effect of spirolactones on toad bladder has shown the specificity and competitive nature of the antagonism to aldosterone. Partial inhibition of the response to aldosterone occurred at a spirolactone SC 14266 to aldosterone ratio of 1000:1 and complete inhibition at 5000:1. That spirolactone inhibits the effect of aldosterone on glucose metabolism as well as that on Na^+ transport would suggest that the decrease in $^{14}CO_2$ from [1-^{14}C] — [6-^{14}C]glucose is related to the stimulation of Na^+ transport. From the results described here it is clear that a 1000-fold excess of spirolactone SC 14266, which partially blocks the stimulation of Na^+ transport by aldosterone, also partially blocks the effect of aldosterone on glucose metabolism. At a 2000-fold excess the effect of aldosterone to decrease $^{14}CO_2$ from [1-^{14}C] — [6-^{14}C]glucose is almost eliminated.

Similar results were obtained when the competition between spirolactone SC 14266 and dexamethasone was tested at ratios of 500:1 and then 2000:1. Dexamethasone was used because of its presumed potent glucocorticoid activity, although it also has mineralocorticoid activity in toad bladder. HANDLER *et al.*[19] have reported that spirolactones inhibited the effect of dexamethasone on $^{14}CO_2$ evolution from [6-^{14}C]glucose but did not inhibit the dexamethasone-induced decrease in $^{14}CO_2$ from [1-^{14}C] — [6-^{14}C]glucose. They used a competition ratio of 625:1. Our results show graded inhibition of the effects of dexamethasone at ratios of 500:1 and 2000:1. The reason for this discrepancy is not clear.

The second test made use of the fact that aldosterone and deoxycorticosterone are more potent in the stimulation of Na^+ transport in toad bladder than cortisol[2,3], which is presumed to be a more active glucocorticoid. When tested for their relative potencies in decreasing $^{14}CO_2$ from [1-^{14}C] — [6-^{14}C]glucose, aldosterone and deoxycorticosterone were found to be more potent than cortisol. When the concentration of these steroids was $5 \cdot 10^{-9}$ M, cortisol was without effect on glucose metabolism, while at this concentration both aldosterone and deoxycorticosterone still exerted definite effects.

Finally, one steroid which is inactive on Na^+ transport, cortisone, and another

very weakly active steroid, cortexolone, were tested. These two steroids at concentrations of $1 \cdot 10^{-7}$ M, like progesterone in a previous study, were not found to have a significant effect upon the evolution of $^{14}CO_2$ from [1-^{14}C]- and [6-^{14}C]glucose. Thus the evidence presently available suggests that ability of a steroid to inhibit the hexose-monophosphate shunt pathway is closely paralleled by the mineralocorticoid ability of the steroid.

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