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Corticosteroids and Ascorbic Acid Transport in Adrenal Cortex *in vitro*

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We have previously reported (Sharma, Johnstone & Quastel, 1963) that the uptake of [^{14}C]-ascorbic acid in guinea-pig adrenal-cortex slices is a process dependent on metabolic energy and inhibited by dinitrophenol and anaerobiosis. The concentration of [^{14}C]ascorbic acid in the tissue may rise to five times that of the medium. Extraction of the tissue with 85% (v/v) ethanol, followed by chromatography and radioautography, showed the presence of a single component whose R_F corresponded to that of ascorbic acid. ACTH,* which depletes the adrenal gland of ascorbic acid *in vivo* (Sayers, Sayers, Liang & Long, 1945, 1946; Long & Fry, 1945), inhibits the active uptake of [^{14}C]-ascorbic acid in guinea-pig adrenal-cortex slices *in vitro*. Moreover, the inhibition was obtained only under conditions leading to adrenal-steroid synthesis. In the absence of glucose, ACTH had no effect on ascorbic acid uptake. It is already well established that added glucose is required to obtain enhanced steroid synthesis by adrenal-cortical tissue *in vitro* in the presence of ACTH (Schonbaum, Birmingham & Saffran, 1956). Adenosine 3',5'-monophosphate, which stimulates adrenal steroid synthesis *in vitro* (Haynes, Koritz & Peron, 1959; Birmingham, Kurlents, Lane, Muhlstock & Traikov, 1960), also inhibited the uptake of [^{14}C]-

ascorbic acid (Sharma *et al.* 1963). As corticosteroids inhibit ascorbic acid uptake (Sharma *et al.* 1963), we investigated further the nature and the specificity of the effects of steroids on ascorbic acid uptake.

MATERIALS AND METHODS

Adrenal-cortex slices. The preparation of guinea-pig adrenal-cortex slices and the incubation procedure that we have used are fully described by Sharma *et al.* (1963). The incubations were carried out in Krebs-Ringer phosphate containing NaCl (134 mM), KCl (5.2 mM), KH_2PO_4 (1.3 mM), MgSO_4 (1.3 mM), CaCl_2 (2.8 mM) and sodium phosphate buffer, pH 7.4 (10 mM). Glucose (10 mM) and thiourea (1 mM) were also added. All experiments were run in duplicate.

Adrenal-cortex homogenate. Adrenal-cortex slices were homogenized in ice-cold Krebs-Ringer phosphate solution (400 mg. wet wt. of tissue in 20 ml. of Krebs-Ringer phosphate) with a Teflon-pestle homogenizer. A 0.5 ml. sample of the homogenate was added to each Warburg manometric vessel. The total volume used was 4.0 ml.

Influx experiments. The influx of [^{14}C]ascorbic acid was measured as described by Sharma *et al.* (1963). When measuring uptake of [^{14}C]ascorbic acid it is necessary to add thiourea (1 mM) to the incubation medium to prevent oxidation of ascorbic acid in the adrenal tissue. This is not necessary for brain slices.

Efflux experiments. The adrenal-cortex slices, after pre-incubation as described by Sharma *et al.* (1963), were

* Abbreviation: ACTH, adrenocorticotrophic hormone.

incubated aerobically (in O_2) at 37° in the presence of [$1-^{14}C$]ascorbic acid for 60 min. The vessels were then placed in crushed ice.

The slices were rinsed in ice-cold Krebs–Ringer medium and transferred to another set of vessels containing a fresh portion of 3 ml. of Krebs–Ringer phosphate medium. This new medium is the same as that above but no [$1-^{14}C$]ascorbic acid was added. The vessels were regassed and reincubated at 37° . After given intervals of time, the vessels were removed from the bath and a sample (0.2 ml.) of the fluid was plated quickly on an aluminium planchet. The vessels were regassed after each sample had been taken out and the incubation was continued. Each sampling time took 5–6 min. Finally the vessels were placed in crushed ice, and the slices were rinsed, homogenized and extracted with 80% (v/v) ethanol as described by Sharma *et al.* (1963).

Dialysis. After incubation at 37° the homogenates were dialysed in a cellophan dialysing bag ($\frac{3}{4}$ in. diam.) containing four to six glass beads. The bag was placed in a conical flask containing 600 ml. of Krebs–Ringer phosphate solution without Ca^{2+} ions at pH 7.4. To agitate the dialysing solution, a magnetic stirrer was used. The dialysing sac was rotated approx. 12 times/min. at 4° for 24 hr. The dialysing solution in the conical flask was changed five times during the dialysis.

The dialysis residue was plated on weighed planchets, dried, weighed and its radioactivity measured. A control experiment with the radioactive substance without tissue was also carried out.

Steroid estimation. The steroids were estimated by a procedure based on methods used by Saffran & Schally (1955) and Elliott, Birmingham, Schally & Schonbaum (1954). After incubation, the medium was divided into two equal portions: one portion (A) was used to estimate the steroid concentration and the other (B) was used as a source of steroid synthesized *in situ* for further experiments. Medium incubated without tissue served as a control and was treated in the same way as that for the medium in which tissue had been incubated. A 0.4 ml. sample of the medium was transferred into each of two glass-stoppered centrifuge tubes of 2.5 ml. capacity. The contents of each tube were extracted with methylene dichloride (3×1 ml.) by shaking and centrifuging. The combined methylene dichloride extracts were evaporated at room temperature in a current of pure N_2 .

When the tubes were completely dry, 1.0 ml. of methanol was added to tube A. The tube was stoppered, shaken well and kept for 30 min. at room temperature. Part of the solution was transferred to a quartz microcuvette and readings were taken at 225, 240, 255 and 260 $m\mu$ in a Beckmann model DU spectrophotometer. The steroids have a maximum absorption peak at 240 $m\mu$. When no peak at about 240 $m\mu$ was obtained, interfering substances were probably present, and the readings were discarded. This was a rare occurrence. The formula suggested by McKerns & Nordstrand (1955) has been used for the calculation of the amount of steroid present.

Resuspension of the steroid in tube B was made in 50% (v/v) ethanol. A 0.02 ml. sample of this solution was used in the incubation medium. In the experiments in which steroids dissolved in ethanol were used, the effect of 0.02 ml. of 50% ethanol was examined separately. All steroids used, including [$4-^{14}C$]cortisone, were made up in 50% ethanol and the final ethanol concentration in the incubation medium was 1% (v/v). [$1-^{14}C$]Ascorbic acid was freshly prepared for each experiment and dissolved in 10 mM-thiourea (the final concentration of thiourea in the incubation medium was 1 mM).

RESULTS

Effects of sodium ions and of ouabain on ascorbic acid uptake. It has become increasingly apparent that Na^+ ions are required for the active transport of a number of amino acids and carbohydrates in a variety of cell types (Ricklis & Quastel, 1958; Csaky & Thale, 1960; Bihler & Crane, 1962; Takagaki, Hirano & Nagata, 1959; Abadom & Scholefield, 1962). The active transport of [$1-^{14}C$]ascorbic acid in guinea-pig adrenal-cortex slices is also dependent on Na^+ ions, 50% inhibition of transport being observed when the sodium chloride is replaced by choline chloride (Table 1). When 50% of the sodium chloride is replaced by choline chloride, inhibition of ascorbic acid uptake is not as extensive but it is still significant. In the absence of Na^+ ions, ascorbate transport is as effectively inhibited as in the

Table 1. *Sodium ion requirement for the uptake of [$1-^{14}C$]ascorbic acid by guinea-pig adrenal-cortex slices*

Guinea-pig adrenal-cortex slices were preincubated in Krebs–Ringer phosphate medium, pH 7.4, containing glucose (10 mM) at 37° for 45 min., and then incubated in a fresh medium, pH 7.4, containing glucose (10 mM) and [$1-^{14}C$]ascorbic acid (0.1 mM; 50 000 counts/min./ml. of incubation medium) at 37° for 60 min. Choline chloride replaced NaCl when this was omitted from the medium. Substances also present during this incubation were: KCl (5.2 mM), $CaCl_2$ (2.8 mM), KH_2PO_4 (1.3 mM), $MgSO_4$ (1.3 mM) and thiourea (1 mM). The final volume was 1 ml. The gas phase was 100% O_2 . Each value reported in this and subsequent Tables is the mean $\pm$ S.D. of at least four separate determinations.

Concn. of additions to final incubation medium (mM)			Uptake of [$1-^{14}C$]ascorbic acid		
Choline chloride	NaCl	Ouabain	Counts/min./100 mg. wet wt. of tissue	Tissue [$1-^{14}C$]ascorbic acid medium [$1-^{14}C$]ascorbic acid concentration ratio	
					Q_{O_2}
0	134	0	18 500 $\pm$ 1 500	5.0	3.4
0	134	0.01	9 600 $\pm$ 800	2.5	3.1
67	67	0	13 600 $\pm$ 1 100	3.6	3.0
134	0	0	9 900 $\pm$ 800	2.6	3.2

Table 2. Uptake of [^{14}C]ascorbic acid by guinea-pig adrenal-cortex slices in the presence of steroid hormones

The conditions were as given in Table 1. All flasks contained 134 mM-NaCl. Steroids were dissolved in 50% ethanol to give a final concn. of 0.1 mM, and 0.02 ml. of the ethanolic solution was added to each vessel. The total volume was 1.0 ml. A control with the same concentration of ethanol was carried out with each experiment.

Steroid (0.1 mM)	Counts/min./100 mg. wet wt. of tissue	Uptake of [^{14}C]ascorbic acid		Q_{O_2}
		medium [^{14}C]ascorbic acid	Tissue [^{14}C]ascorbic acid concentration ratio	
None	19 000 $\pm$ 1 500		5.1	3.0
Hydrocortisone	13 000 $\pm$ 1 100		3.5	2.5
Oestradiol	19 000 $\pm$ 1 500		5.1	2.8
Testosterone	19 000 $\pm$ 1 500		5.1	3.5
Progesterone	17 000 $\pm$ 1 400		4.5	3.6
Pregnenalone	19 000 $\pm$ 1 500		5.1	3.9
Dehydroisoandrosterone	17 000 $\pm$ 1 400		4.5	2.7
Aldosterone	13 000 $\pm$ 1 100		3.4	3.0

absence of metabolic energy (Sharma *et al.* 1963). There is an inhibitory effect of ouabain on ascorbic acid uptake (Table 1) which no doubt is related to its known effect on Na^+ ion transport at cell membranes.

Specificity of steroids inhibiting uptake of [^{14}C]ascorbic acid. Of the steroids normally produced by the adrenal cortex that were examined, only those that possess a ketol ($\text{HO}\cdot\text{CH}_2\cdot\text{CO}-$) side chain at C-17 of the steroid nucleus inhibit the uptake of [^{14}C]ascorbic acid (Table 2). We have shown (Sharma *et al.* 1963) that cortisone, corticosterone and deoxycorticosterone are as effective as hydrocortisone in inhibiting the uptake of [^{14}C]ascorbic acid. Progesterone, pregnenalone and dehydroisoandrosterone are all produced by the adrenal cortex but do not inhibit ascorbate uptake in guinea-pig-adrenal tissue.

Testosterone and oestradiol, even at concentrations considerably greater than that of hydrocortisone, have little effect on the uptake of [^{14}C]ascorbic acid by adrenal-cortex slices *in vitro* (Fig. 1).

When adrenal-cortical tissue is preincubated with cortisone, rinsed and reincubated with [^{14}C]ascorbic acid in fresh medium, in the absence of any further steroid, a diminished uptake of [^{14}C]ascorbic acid is still observed. The results in Table 3 show that the uptake of [^{14}C]ascorbic acid is greatly decreased in a tissue slice preincubated with cortisone. No such diminution is observed when the tissue is preincubated with testosterone. The fact that cortisone inhibits ascorbic acid uptake in a tissue preincubated with the steroid probably indicates that some of the corticosteroid remains associated with the tissue.

Uptake of [^{14}C]cortisone by guinea-pig adrenal-cortex slices. The results presented in Table 4 show that [^{14}C]cortisone is taken up by adrenal-cortical

Fig. 1. Uptake of [^{14}C]ascorbic acid and [^{14}C]serine by guinea-pig adrenal-cortex slices in the presence of various concentrations of steroid hormones. The conditions were as described in Table 2. The [^{14}C]ascorbic acid concn. was 0.1 mM (50 000 counts/min./ml.), and the [^{14}C]serine concn. was 0.1 mM (50 000 counts/min./ml.). O, [^{14}C]ascorbic acid and oestradiol; ▲, [^{14}C]ascorbic acid and testosterone; ●, [^{14}C]ascorbic acid and hydrocortisone; △, [^{14}C]serine and hydrocortisone.

tissue. This process is clearly distinct from that observed with [^{14}C]ascorbic acid as it is not affected by either anaerobic conditions or by the presence of ouabain. The uptake is dependent on temperature and is proportional to the medium concentration of cortisone between 0.1 and 0.5 mM.

The uptake of [^{14}C]cortisone appears to be due to a binding of cortisone to tissue constituents rather than to an active transport system. When homogenates of the adrenal cortex are incubated with [^{14}C]cortisone and then dialysed, a considerable portion of the radioactivity remains

associated with the dialysis residue. [4-¹⁴C]-Cortisone, in the absence of adrenal-cortical tissue, is not retained by the dialysis sac. It is well known that corticosteroids are bound by proteins (Bellamy, Phillips, Jones & Leonard, 1962).

Transport of amino acids in guinea-pig adrenal-cortex slices in the presence of corticosteroids and adrenocorticotrophic hormone. The results in Table 5 show that the uptake of [¹⁴C₃]serine or α-amino-[1-¹⁴C]isobutyrate is not affected by either ACTH or cortisone under conditions that cause a marked inhibition of ascorbic acid uptake.

Concentrations of hydrocortisone up to 1 mM scarcely affect [¹⁴C₃]serine uptake, in contrast with

the marked inhibition of [1-¹⁴C]ascorbic acid uptake (Fig. 1). [¹⁴C₃]Serine (0.1 mM) is concentrated by adrenal-cortex slices, a ¹⁴C ratio (cell water/medium) of 3.0 being obtained after 60 min. of incubation. With α-aminoisobutyrate (0.1 mM) the concentration ratio is 2.0. A number of amino acids (all at 0.1 mM), namely proline, glycine, glutamate, aspartate, methionine, phenylalanine, valine, leucine, γ-aminobutyrate and 5-hydroxytryptophan, attained a concentration ratio of about 1.

The uptake of [¹⁴C₃]serine is inhibited by ouabain and by anaerobiosis, but α-amino[1-¹⁴C]isobutyrate uptake is apparently not affected. These results suggest that, of the amino acids examined, only the

Table 3. *Uptake of [1-¹⁴C]ascorbic acid in adrenal-cortex slices preincubated with steroid hormones*

Tissue slices were preincubated for 45 min. at 37° in Krebs-Ringer phosphate medium, pH 7.4, containing glucose (10 mM) and the steroids. The slices were rinsed thoroughly in cold Krebs-Ringer phosphate medium and reincubated in a fresh medium without any further addition of steroid and containing [1-¹⁴C]ascorbic acid (0.1 mM; 50 000 counts/min./flask). The gas phase was O₂ and the incubation time was 60 min. at 37°. All other conditions were as given in Table 2.

Addition to preincubation medium	Uptake of [1- ¹⁴ C]ascorbic acid		Q _{O₂}
	Counts/min./100 mg. wet wt. of tissue	Tissue [1- ¹⁴ C]ascorbic acid medium [1- ¹⁴ C]ascorbic acid concentration ratio	
None	17 200 ± 1 400	4.8	3.0
Cortisone (1.0 mM)	9 000 ± 700	2.5	2.5
Testosterone (1.0 mM)	17 000 ± 1 400	4.7	2.4

Table 4. *Uptake of [4-¹⁴C]cortisone by guinea-pig adrenal-cortex slices*

The preincubation time was 45 min., and the final incubation was 60 min. at 37°. The conditions were as given in Table 2.

Addition	Temp.	Gas phase	Concn. of [4- ¹⁴ C]cortisone added (mM)	Uptake of [4- ¹⁴ C]cortisone (μmoles/g. wet wt. of tissue)
None	0°	O ₂	0.1	0.06
None	37	O ₂	0.1	0.28
None	37	O ₂	0.5	1.30
None	37	N ₂	0.1	0.29
Ouabain (0.1 mM)	37	O ₂	0.1	0.27
Ascorbic acid (10 mM)	37	O ₂	0.1	0.19

Table 5. *Amino acid uptake by guinea-pig adrenal-cortex slices in the presence of adrenocorticotrophic hormone and corticosteroids*

The preincubation time was 45 min. and the final incubation was 60 min. at 37°. The conditions were as given in Table 2. The ACTH concn. was 0.2 unit/ml., the cortisone concn. was 0.1 mM, and the test substance concn. was 0.1 mM (radioactivity 50 000 counts/min./ml.).

Addition	Test substance	Uptake of test substance (counts/min./100 mg. wet wt. of tissue)
None	L-[¹⁴ C ₃]Serine	13 700 ± 1 100
ACTH	L-[¹⁴ C ₃]Serine	13 700 ± 1 100
Cortisone	L-[¹⁴ C ₃]Serine	13 600 ± 1 100
None	α-Amino[1- ¹⁴ C]isobutyrate	8 000 ± 600
ACTH	α-Amino[1- ¹⁴ C]isobutyrate	8 000 ± 600
Cortisone	α-Amino[1- ¹⁴ C]isobutyrate	7 800 ± 600
None	[1- ¹⁴ C]Ascorbic acid	19 000 ± 1 500
Cortisone	[1- ¹⁴ C]Ascorbic acid	13 600 ± 1 100

uptake of [^{14}C]serine is an energy-dependent process in the adrenal cortex *in vitro*.

The fact that ACTH and cortisone do not inhibit the uptake of [^{14}C]serine shows that their effects on ascorbic acid uptake are due to their association with a specific transport system rather than with a general transport phenomenon.

Uptake of [$^{14}\text{C}_6$]glucose and [$2,3\text{-}^{14}\text{C}_2$]succinate in the presence of adrenocorticotrophic hormone. With [$^{14}\text{C}_6$]glucose or [$2,3\text{-}^{14}\text{C}_2$]succinate as substrate, the amount of radioactivity found in the ethanol-soluble fraction of the tissue is unaltered in presence of ACTH (Table 6). Moreover, the production of $^{14}\text{CO}_2$ from [^{14}C]glucose and [$6\text{-}^{14}\text{C}$]glucose is not affected by cortisone.

Steroids and ascorbic acid uptake in other tissues. Rat-brain-cortex slices, as well as guinea-pig adrenal-cortex slices, bring about the active transport of [$1\text{-}^{14}\text{C}$]ascorbic acid (Sharma *et al.* 1963). The present results show that guinea-pig-ovary slices are also able to transport [$1\text{-}^{14}\text{C}$]ascorbic acid against a concentration gradient. Under the present experimental conditions a con-

centration ratio of 3 for the cell/medium distribution of [$1\text{-}^{14}\text{C}$]ascorbic acid was obtained after 1 hr. incubation with 0.1 mM- $[1\text{-}^{14}\text{C}]$ ascorbic acid (Table 7). As with brain and adrenal-cortex slices, the uptake of [$1\text{-}^{14}\text{C}$]ascorbic acid in guinea-pig-ovary slices is decreased under anaerobic conditions.

The effect of steroids on [$1\text{-}^{14}\text{C}$]ascorbic acid uptake in brain and ovary slices differs from that obtained with adrenal-cortex slices. Oestradiol, testosterone and progesterone, as well as the adrenal-cortical steroids, inhibit the uptake of ascorbic acid in rat-brain-cortex slices. Most of these steroids also inhibit the uptake of [$1\text{-}^{14}\text{C}$]ascorbic acid in ovary slices (Table 7). ACTH, however, has no effect on ascorbic acid uptake in brain or ovary preparations. The steroids that inhibit ascorbic acid uptake at a concentration of 0.1 mM have no effect on the respiratory activity of the rat-brain slices or the guinea-pig-ovary slices. Deoxycorticosterone and corticosterone, but not hydrocortisone or cortisone, inhibit the respiration of rat-brain slices at concentrations of 0.5 mM. In adrenal-cortex preparations, a concentration of 0.1 mM-corticosteroid, which inhibits ascorbic acid uptake, has no effect on the respiratory activity.

The uptake of [$1\text{-}^{14}\text{C}$]ascorbic acid in brain and ovary slices, therefore, is not specifically inhibited by adrenal-cortical steroids with a ketol side chain at C-17. All the steroids examined, whether of ovarian, testicular or adrenal origin, have similar inhibitory effects at equivalent concentrations on ascorbic acid uptake in brain and ovary.

Action of corticosteroids synthesized in situ on ascorbic acid uptake. It has been shown that the uptake of [$1\text{-}^{14}\text{C}$]ascorbic acid is inhibited by ACTH under conditions that promote steroid synthesis and also by certain known corticosteroids. If the steroids produced in response to ACTH are the

Table 6. *Uptake of radioactivity from [^{14}C]glucose and [^{14}C]succinate by adrenal-cortex slices*

The conditions were as given in Table 2, except that ascorbic acid was omitted. The radioactivity added in all cases was 50 000 counts/min./ml. of incubation medium.

Addition to final incubation medium	Concn. of ACTH (unit/ml. of incubation medium)	Uptake of ^{14}C (counts/min./100 mg. wet wt. of tissue)
[$^{14}\text{C}_6$]Glucose (2.0 mM)	0	2400
[$^{14}\text{C}_6$]Glucose (0.1 mM)	0	3300
[$^{14}\text{C}_6$]Glucose (2.0 mM)	0.2	2400
[$2,3\text{-}^{14}\text{C}_2$]Succinate (2.0 mM)	0	2300
[$2,3\text{-}^{14}\text{C}_2$]Succinate (0.1 mM)	0	3700
[$2,3\text{-}^{14}\text{C}_2$]Succinate (2.0 mM)	0.2	2200

Table 7. *Uptake of [$1\text{-}^{14}\text{C}$]ascorbic acid by rat-brain-cortex and guinea-pig-ovary slices*

The [$1\text{-}^{14}\text{C}$]ascorbic acid concn. was 0.1 mM, and the steroid concn. was 0.1 mM. The conditions of the experiment were as given in Table 2. There was no preincubation period with rat-brain-cortex slices.

Steroid	Uptake of [$1\text{-}^{14}\text{C}$]ascorbic acid			
	By rat-brain-cortex slices		By guinea-pig-ovary slices	
	Counts/min./100 mg. wet wt. of tissue	Tissue [$1\text{-}^{14}\text{C}$]ascorbic acid medium [$1\text{-}^{14}\text{C}$]ascorbic acid concentration ratio	Counts/min./100 mg. wet wt. of tissue	Tissue [$1\text{-}^{14}\text{C}$]ascorbic acid medium [$1\text{-}^{14}\text{C}$]ascorbic acid concentration ratio
None	12 700 $\pm$ 1 000	3.8	11 500 $\pm$ 900	3.0
Hydrocortisone	7 100 $\pm$ 600	1.9	7 600 $\pm$ 600	1.8
Deoxycorticosterone	6 500 $\pm$ 500	1.7	—	—
Cortisone	6 400 $\pm$ 500	1.7	—	—
Corticosterone	6 900 $\pm$ 600	1.8	—	—
Oestradiol	7 600 $\pm$ 600	2.1	7 400 $\pm$ 600	1.8
Testosterone	8 000 $\pm$ 600	2.3	6 800 $\pm$ 600	1.7
Progesterone	10 000 $\pm$ 800	2.7	—	—

inhibitors of ascorbic acid uptake, it should be possible to demonstrate that the steroids produced *in situ* are capable of inhibiting the uptake of ascorbic acid by adrenal-cortex slices.

The results in Table 8 demonstrate the well-known fact that ACTH stimulates the production of steroids in guinea-pig adrenal cortex. A two- to three-fold increase in corticosteroids is usually obtained after 2 hr. of incubation with 0.2 unit of ACTH/ml.

To estimate steroid synthesis under conditions identical with those used for measurements of ascorbic acid uptake, we have examined the effects of thiourea on steroid production. The results in Table 8 clearly show that 1 mM-thiourea has little effect on corticosteroid production in the presence or absence of ACTH.

Steroids formed *in situ* in response to ACTH are considerably more active than the individual synthetic corticosteroids tested in bringing about inhibitions of ascorbic acid uptake (Table 9).

Table 8. *Steroid production in guinea-pig adrenal-cortex slices in the presence and absence of adreno-corticotrophic hormone and thiourea*

The ACTH concn. was 0.2 unit/ml. of incubation medium, and the thiourea concn. was 1.0 mM. Other conditions of incubation were as given in Table 2. Adrenal-cortex slices were preincubated for 45 min. and then incubated for 2 hr. with 10 mM-glucose at pH 7.4 and 37°. The steroids in the medium were extracted with methylene dichloride and measured by the ultraviolet-absorption method.

Additions	Steroid production ($\mu\text{g.}/100$ mg. wet wt. of tissue)
None	3.8
Thiourea	4.0
ACTH	9.2
Thiourea + ACTH	8.2

To obtain and test the steroids synthesized *in situ* the procedure adopted was as follows. Equal weights of guinea-pig adrenal-cortex slices were incubated for 2 hr. with and without ACTH in a normal Krebs-Ringer medium containing glucose (10 mM). The tissue was discarded and the medium was extracted with methylene dichloride (see the Materials and Methods section). Methylene dichloride was evaporated under a stream of nitrogen and the residue redissolved in 50% ethanol. Fresh adrenal-cortex slices were prepared in a fresh incubation medium containing $[1-^{14}\text{C}]$ ascorbic acid and appropriate samples of the ethanol extract were added to give the desired concentration of steroid (see the Materials and Methods section). The ethanol extract from tissue, incubated without ACTH, served as a control, and the volumes of ethanol extract used from this preparation corresponded to those used from the ACTH-containing preparation. Uptake of ascorbic acid was then measured in the usual way. The results in Table 9 show that the steroids produced *in situ* are much more effective than an equivalent concentration of cortisone in inhibiting the uptake of $[1-^{14}\text{C}]$ ascorbic acid. Even at a concentration of $5\mu\text{g.}/\text{ml.}$ (equivalent to 0.017 mM-cortisone) there is an appreciable decrease in uptake, corresponding to that obtained with $30\mu\text{g.}$ of cortisone. At concentrations below $30\mu\text{g.}/\text{ml.}$ (i.e. 0.1 mM) none of the commercially available corticosteroids produces a significant inhibition of uptake of $[1-^{14}\text{C}]$ ascorbic acid.

An ethanol extract of the medium incubated without ACTH has no significant effect on the uptake of $[1-^{14}\text{C}]$ ascorbic acid. These results therefore support the hypothesis that the substances, presumably corticosteroids, produced in response to ACTH are the most effective inhibitors of ascorbic acid uptake.

All the commercial corticosteroids we have tested with a ketol side chain at C-17 were found to give

Table 9. *Effect of steroids formed in situ on $[1-^{14}\text{C}]$ ascorbic acid uptake by guinea-pig adrenal-cortex slices*

The preincubation time was 45 min., and the final incubation was 60 min. at 37°. The conditions were as given in Table 2.

Source of steroid	Concn. of steroid ($\mu\text{g.}/\text{ml.}$)	Uptake of $[1-^{14}\text{C}]$ ascorbic acid	
		Counts/min./100 mg. wet wt. of tissue	Tissue $[1-^{14}\text{C}]$ ascorbic acid medium $[1-^{14}\text{C}]$ ascorbic acid concentration ratio
None	0	$19\,400 \pm 1\,500$	5.3
Formed <i>in situ</i> *	30	$10\,000 \pm 800$	2.5
	15	$14\,000 \pm 1\,100$	3.6
	5	$15\,000 \pm 1\,200$	3.8
	5	$15\,000 \pm 1\,500$	5.2
Cortisone	30	$14\,000 \pm 1\,100$	3.7
	15	$18\,700 \pm 1\,500$	5.0
	5	$19\,000 \pm 1\,500$	5.2
	5	$19\,000 \pm 1\,500$	5.2
Control medium	0	$18\,400 \pm 1\,500$	5.0
	0	$22\,400 \pm 1\,500$	5.7
	0	$20\,000 \pm 1\,500$	5.3

* The quantities of steroid formed *in situ* are given in equivalents of cortisone (see the Materials and Methods section).

almost the same amount of inhibition of ascorbic acid uptake at the concentrations used. The fact that the steroids synthesized *in situ* are appreciably more effective than any of the commercially available products at equivalent concentrations indicates that either (a) a mixture of steroids is more effective than a single compound, or (b) a particular steroid synthesized *in situ* is a far more effective inhibitor than any of the compounds examined. To test the first possibility a mixture of four steroids, namely cortisone, hydrocortisone, corticosterone and deoxycorticosterone, was used at a final total concentration of 0.2 mM, each steroid concentration in the mixture being 0.05 mM, and its effects were compared with that of each of the four at 0.2 mM. The results in Table 10 show that a mixture of steroids is more effective in bringing about inhibitions of uptake of [1-¹⁴C]ascorbic acid than is each steroid examined separately. However, even a mixture of the steroids is considerably less effective than equivalent concentrations of the steroids synthesized *in situ*. These results suggest that a particular corticosteroid, which has so far not been examined in the present work, is more effective than other corticosteroids in blocking the uptake of [1-¹⁴C]ascorbic acid in adrenal-cortex slices.

Efflux of ascorbic acid. ACTH is known to deplete the adrenal gland of ascorbic acid *in vivo*. The adrenal gland is not known to synthesize ascorbic acid. Presumably, under normal conditions, the ascorbic acid in the tissue is at a steady state, the uptake from the blood stream being balanced by a corresponding loss from the tissue. It is known that in certain transport systems inhibition of the uptake will result in an efflux of material from the cell (Johnstone & Scholefield, 1959; Gonda & Quastel, 1962).

We have now been able to demonstrate that conditions that bring about a decreased uptake of [1-¹⁴C]ascorbate also bring about a depletion of tissue

[1-¹⁴C]ascorbate. Thus the addition of ACTH or corticosteroids to adrenal tissue preincubated with [1-¹⁴C]ascorbic acid increases the loss of [1-¹⁴C]ascorbic acid from the tissue.

In these experiments the tissue slices were preincubated with 0.1 mM-[1-¹⁴C]ascorbic acid, then rinsed and placed in fresh medium without further ascorbic acid but containing, where indicated, ACTH or corticosteroids.

It is to be expected that some [1-¹⁴C]ascorbic acid will be lost from all the tissues on reincubation in fresh medium containing no ascorbic acid. The efflux ceases when the rate of loss from the tissue and the rate of transport back into the tissue become balanced, i.e. when a new steady state is reached. If, however, the transport back into the tissue is blocked by ACTH or corticosteroids, the efflux of radioactivity will continue for a longer time.

The results presented in Fig. 2 show the effects of ACTH and cortisone on the rate of efflux of [1-¹⁴C]ascorbic acid from adrenal-cortex slices. It is apparent that initially (during the first 30 min.) the amount of radioactivity lost is approximately the same under all conditions. In the presence of ACTH or cortisone the efflux continues, whereas in their absence a steady state is attained. Similarly hydrocortisone increases the loss of [1-¹⁴C]ascorbic acid from the tissue, but oestradiol and testosterone have no effect (Fig. 3).

Anaerobiosis, which also inhibits transport of [1-¹⁴C]ascorbic acid in adrenal-cortex slices, enhances the loss of [1-¹⁴C]ascorbate from the tissue (Fig. 3). Clearly, therefore, under conditions which inhibit the transport of ascorbic acid, an increased loss of [1-¹⁴C]ascorbate from tissue preincubated with [1-¹⁴C]ascorbic acid can be attained. These conditions are analogous to conditions *in vivo* and indicate that ACTH and corticosteroids 'deplete' the adrenal gland of ascorbic acid *in vitro* as well as *in vivo*.

Table 10. Inhibition of [1-¹⁴C]ascorbic acid uptake in guinea-pig adrenal-cortex slices by a mixture of steroids

The [1-¹⁴C]ascorbic acid concn. was 0.1 mM. The preincubation time was 45 min., and the final incubation was 60 min. at 37°. The commercial mixture contained hydrocortisone, cortisone, corticosterone and deoxycorticosterone, each at 0.05 mM. The conditions were as given in Table 2.

Addition	Concn. of steroid (mM)	Uptake of [1- ¹⁴ C]ascorbic acid	
		Counts/min./100 mg. wet wt. of tissue	Tissue [1- ¹⁴ C]ascorbic acid medium [1- ¹⁴ C]ascorbic acid concentration ratio
None	0	19 200 ± 1 500	5.3
Formed steroids	0.08	10 000 ± 800	2.5
Commercial mixture	0.2	10 000 ± 800	2.5
Hydrocortisone	0.2	14 400 ± 1 100	3.7
Cortisone	0.2	13 000 ± 1 100	3.4
Corticosterone	0.2	14 400 ± 1 100	3.7
Deoxycorticosterone	0.2	12 800 ± 1 000	3.4

Fig. 2. Efflux of [1-¹⁴C]ascorbic acid from guinea-pig adrenal-cortex slices in the presence of ACTH and cortisone. The tissue was not preincubated in Krebs-Ringer medium in these experiments because, after prolonged incubation, the tissue does not synthesize extra steroids in response to ACTH. The tissue, after removal from the animals, was incubated for 1 hr. at 37° in O₂ in a Krebs-Ringer medium containing [1-¹⁴C]ascorbic acid (0.1 mM; 50 000 counts/min./ml. of medium), glucose (10 mM) and thiourea (1 mM). The tissue was then removed from the flasks, rinsed in cold iso-osmotic Krebs-Ringer medium and reincubated in fresh Krebs-Ringer glucose medium containing ACTH, or cortisone, but without further addition of ascorbic acid. Samples of the medium were taken at intervals to estimate radioactivity. ▲, Control; ○, ACTH (0.5 unit/100 mg. wet wt. of tissue) added; ●, cortisone (0.2 mM) added.

DISCUSSION

The effect of ACTH on ascorbic acid depletion of the adrenal gland forms the basis of a well-known assay for ACTH (Sayers, Sayers & Woodbury, 1948). It has hitherto been assumed that ACTH acts directly on the gland and that the primary mechanism is to cause a release of the tissue ascorbate. An increased efflux can be obtained by two separate mechanisms: (a) a stimulation of efflux without any alteration of influx, or (b) an inhibition of influx resulting in an increased net efflux. The results obtained are consistent with the latter mechanism. When ACTH (or corticosteroids) is added to the adrenal-cortex slices, there is an increased amount of [1-¹⁴C]ascorbate lost from the tissue. The difference between the amounts of ascorbic acid lost from the tissue in the presence and absence of ACTH (or corticosteroids) becomes progressively larger with time of incubation (Figs. 2 and 3), a steady state being reached in the absence of ACTH (or corticosteroids) after 30 min. If efflux were stimulated by ACTH or corticosteroids, the amounts of ascorbic acid lost from

Fig. 3. Efflux of [1-¹⁴C]ascorbic acid in the presence of steroid hormones. Guinea-pig adrenal-cortex slices were preincubated for 45 min. in Krebs-Ringer phosphate medium, pH 7.4, containing glucose (10 mM), in an atmosphere of O₂. The slices were then transferred to fresh medium of the same composition but containing also [1-¹⁴C]ascorbic acid (0.1 mM; 50 000 counts/min./ml.) and incubated for 60 min. After 60 min. the slices were removed and rinsed in cold iso-osmotic Krebs-Ringer medium and placed in fresh medium of the same composition as above but without ascorbic acid. Steroids were added where indicated. For anaerobic incubation, an atmosphere of N₂ instead of O₂ was used in the final incubation. The medium was assayed for radioactivity. ■, Anaerobic incubation (in N₂); ●, hydrocortisone (0.2 mM) added; ○, control; ▲, testosterone (0.2 mM) added; △, oestradiol (0.2 mM) added.

tissue in the early stage of the experiment would be expected to be higher than that found in the absence of ACTH or corticosteroids. The results given by Sharma *et al.* (1963) and in the present work indicate that the effect of ACTH is mediated by corticosteroids produced in its presence and that the primary mode of action is an inhibition of transport of ascorbic acid into the tissue.

Ascorbic acid in the adrenal gland in mammals is present at a concentration about 100 times that in the plasma. As ascorbic acid is not synthesized by the adrenal gland, the high concentration of ascorbic acid in the adrenal may be the result of an active transport mechanism. It has been shown that transport of [1-¹⁴C]ascorbic acid in guinea-pig adrenal-cortex slices *in vitro* is an active process dependent on metabolic energy. The transport is inhibited by anaerobiosis or by dinitrophenol. It is also dependent on the presence of Na⁺ ions, and ouabain, which inhibits Na⁺ ion transport across a variety of cell membranes, also inhibits the transport of [1-¹⁴C]ascorbic acid. Of a variety of steroids

examined, only adrenal-cortical steroids with a ketol group at C-17 inhibit the uptake of [$1\text{-}^{14}\text{C}$]ascorbic acid in adrenal-cortex slices. Rat-brain-cortex slices and ovary slices, which also actively transport [$1\text{-}^{14}\text{C}$]ascorbic acid, do not show any specificity for the ketol-containing corticosteroids, and are not affected by ACTH. Transport of [$1\text{-}^{14}\text{C}$]ascorbic acid in brain and ovary preparations is inhibited by oestradiol, testosterone and progesterone as well as corticosteroids. In other tissues examined, including liver, pancreas, spleen, diaphragm and testes, active transport of [$1\text{-}^{14}\text{C}$]ascorbic acid was not observed.

To determine whether the corticosteroids specifically inhibit ascorbic acid transport, the effects of the corticosteroids on the transport of other substances were also studied. The uptake of ten amino acids by adrenal-cortex slices was examined. Only serine was found to be transported by an energy-dependent ouabain-sensitive mechanism. ACTH and corticosteroids had no effect on the transport of serine under conditions that brought about a considerable decrease of ascorbic acid transport.

The uptake of radioactivity from [^{14}C]glucose and [$2,3\text{-}^{14}\text{C}_2$]succinate was not affected by ACTH or corticosteroids. $^{14}\text{CO}_2$ production from [$1\text{-}^{14}\text{C}$]glucose and [$6\text{-}^{14}\text{C}$]glucose was not affected by cortisone. Neither ACTH nor corticosteroids inhibited the respiratory activity of the tissues examined at the concentrations tested.

Clearly the corticosteroids specifically inhibit the transport of ascorbic acid in adrenal-cortex slices. We have previously observed that the uptake of [$1\text{-}^{14}\text{C}$]ascorbic acid is inhibited by a medium that had been exposed to ACTH-stimulated slices. Steroids synthesized *in situ* in response to ACTH have now been extracted and their effects on ascorbic acid uptake measured. These steroids are at least three times more effective inhibitors of ascorbate uptake than any of the synthetic corticosteroids so far examined, significant inhibitions being obtained at a concentration of 0.016 mM-steroid. Since the material synthesized *in situ* is not an individual component but presumably a mixture of steroids, a mixture of commercial corticosteroids was prepared and tested. This mixture was not as potent as the mixture of steroids synthesized *in situ*. The high inhibitory activity of the steroids synthesized *in situ* suggests that a particular corticosteroid formed in the presence of ACTH is more effective than others in blocking ascorbate transport in the adrenal cortex. It seems possible that this hypothetical steroid might control ascorbate transport in a manner analogous to the action of aldosterone on ion movements.

It has also been demonstrated that pre-exposure of the adrenal tissue to cortisone decreases the transport of [$1\text{-}^{14}\text{C}$]ascorbic acid. This effect is also

characteristic of the corticosteroids since pre-exposure to testosterone does not alter the transport. [$4\text{-}^{14}\text{C}$]Cortisone appears to be bound to this tissue by a temperature-dependent but energy-independent process.

It is suggested that the corticosteroids inhibit transport of ascorbic acid in adrenal-cortical tissue by their combination with the specific transport site for ascorbic acid. As, of the steroids examined, only corticosteroids with a ketol group at C-17 are effective inhibitors, it is suggested that the ketol group of the steroid combines with this site. The mechanism of depletion of adrenal ascorbic acid by ACTH seems to be due to an inhibition of the active transport of ascorbic acid brought about by the corticosteroids produced in response to ACTH.

SUMMARY

1. The transport of [$1\text{-}^{14}\text{C}$]ascorbic acid in guinea-pig adrenal cortex *in vitro* is a Na^+ ion-dependent ouabain-sensitive process.

2. Of the steroids examined, only corticosteroids with a ketol group at C-17 inhibit the transport of [$1\text{-}^{14}\text{C}$]ascorbic acid in guinea-pig adrenal cortex. A number of other steroids of adrenal, ovarian and testicular origin have no effect.

3. The transport of serine, an energy-dependent and Na^+ ion-dependent process, in guinea-pig adrenal-cortex tissue is not inhibited by corticosteroids. The uptake of a number of other amino acids, glucose and succinic acid is not affected by corticosteroids.

4. The pre-exposure of adrenal-cortex slices to cortisone, but not to testosterone, diminishes [$1\text{-}^{14}\text{C}$]ascorbic acid uptake.

5. [$4\text{-}^{14}\text{C}$]Cortisone is bound by adrenal cortex by a temperature-dependent but energy-independent process.

6. Guinea-pig-ovary slices, as well as rat-brain-cortex slices, transport [$1\text{-}^{14}\text{C}$]ascorbic acid by an energy-dependent process. The uptake of [$1\text{-}^{14}\text{C}$]ascorbic acid in a number of other tissues, including liver, pancreas and spleen, does not appear to be energy-dependent.

7. The transport of [$1\text{-}^{14}\text{C}$]ascorbic acid in rat-brain-cortex slices and guinea-pig-ovary slices is inhibited by corticosteroids as well as by oestradiol and testosterone.

8. Steroids formed *in situ* in response to adreno-corticotrophic hormone inhibit the transport of [$1\text{-}^{14}\text{C}$]ascorbic acid to a greater extent than do equivalent concentrations of commercially available individual steroids or mixtures of the latter.

9. In adrenal-cortex slices preincubated with [$1\text{-}^{14}\text{C}$]ascorbic acid an increased loss of the label is obtained under conditions that have been shown to inhibit transport of ascorbic acid. It is evident that

adrenocorticotrophic hormone and corticosteroids cause an efflux of ascorbic acid from adrenal-cortex slices *in vitro*, this being due to inhibition of active transport into the tissue.

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Metabolism of Palmitate in Sheep

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The metabolic importance of the plasma free fatty acid fraction of circulating lipids, first suggested by the observations of Dole (1956) and Gordon & Cherkas (1956), has been confirmed by measurements of turnover rate based on isotope dilution in man (Laurell, 1957; Frederickson & Gordon, 1958; Havel, 1961), dogs (Bierman, Schwartz & Dole, 1957; Armstrong *et al.* 1961) and rats (Olivecrona, 1962). Single-injection studies with [^{14}C]palmitate or [^{14}C]oleate have formed the basis of most investigations, but Armstrong *et al.* (1961) measured turnover of free fatty acid in dogs by a continuous-infusion technique. In this investigation, as in most others, plasma free fatty acid was treated as a single metabolic fraction and the assumption made that the metabolism of the injected fatty acid (palmitate) was representative of that of the whole plasma free fatty acid fraction. We have measured entry rates of palmitate in sheep by isotope-dilution procedures based on the continuous infusion of [^{14}C]palmitate. This acid was isolated from the other components of plasma free fatty acid by chromatography before measurement

of specific radioactivity. Comparison of the specific radioactivities of plasma palmitate and blood carbon dioxide allowed the contribution of palmitate to total carbon dioxide production to be estimated roughly. The transfer of radioactivity from palmitate into other plasma lipids and certain blood constituents was also followed.

MATERIALS AND METHODS

Animals. Merino ewes and wethers (age 2–3 years) were housed indoors and fed with lucerne chaff (800 g./day). The animals were trained to stand quietly in stocks, sheep failing to respond to training (about 50%) being rejected.

Measurement of rate of entry of palmitate. The general procedure described by Annison & White (1961) for the measurement of glucose entry rates was used. Labelled palmitate in albumin (0.75%) was infused at 1.0 μC , 0.05 $\mu\text{mole/min.}$, without using a priming dose (Armstrong *et al.* 1961). Constancy of plasma FFA* concentration is a prerequisite for entry rate measurements, but FFA concentrations in sheep rise sharply in response even to mild excitement. Disturbance of the animal due to handling or

* Abbreviation: FFA, free fatty acid.