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ALDOSTERONE PRODUCTION BY SHEEP ADRENAL GLANDS *IN VITRO**

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SUMMARY

A novel technique for the preparation of adrenal tissue from large animals (sheep) is presented. Multiple adrenal cortex biopsies are taken using a 2 mm dia. dermatological punch and homogeneous pools of 10 tissue cylinders (1 from each gland) were incubated. Aldosterone, corticosterone and cortisol in the incubation medium were measured by double isotope derivative dilution assay. The mean within-incubation coefficient of variation of aldosterone production was 15.8%, whilst between days, unstimulated aldosterone production ranged from 0.02–0.25 μg/100 mg tissue/2 h. Aldosterone production was stimulated by ACTH, angiotensin II and potassium ions, in a manner similar, but at higher doses, to that reported for sheep *in vivo*. Serotonin (10^{-3} mol/l) inhibited aldosterone production, lower concentrations having no effect. Substrate levels of progesterone, DOC and corticosterone (25 μg/ml) stimulated aldosterone output whilst cortisol inhibited aldosterone production. The incubation system described is simple with a high degree of reproducibility and has the advantage that up to 30 comparable incubation flasks can be prepared for any incubation, thus providing a simple technique for testing possible agonists or antagonists of steroid production.

INTRODUCTION

Classically, aldosterone secretion was believed to be under the control of four factors: ACTH, angiotensin II (AII), plasma [K] and [Na]. However, in recent years, the possibility of existence of another controlling factor, perhaps of neural origin, has been suggested in sheep[1–3]. To assess the biological activity of fractions prepared during the isolation of such a factor, it is necessary to have a bioassay system, whereby many samples can be screened rapidly and simultaneously.

The sheep cervical adrenal autotransplant preparation[4] has been used at this Institute for the past 15 years and much information about the biosynthesis and control of aldosterone has been obtained. Unfortunately, this preparation is not practical for the large-scale testing of extracts, because only a few samples could be tested on any one day. Similarly, although this preparation has been used with great success to study the conversion of [^{3}H]-corticosterone to aldosterone during Na deficiency[5], again the number of experiments which can be performed is very limited.

An *in vitro* technique for the incubation of sheep adrenals would be of great use for the large-scale bioassay of extracts, and also to investigate aldosterone biosynthetic pathways during Na deficiency. This paper describes a method for the preparation of sheep adrenal tissue for incubation and the response of the preparation to known agonists to aldosterone production.

EXPERIMENTAL

Preparation of tissue and incubation procedure. Sheep adrenal glands were collected within 15–20 min of slaughter from local abattoirs. The adrenals were placed into modified Krebs–Ringer buffer, containing 4 mmol/l of potassium and 2 g/l of glucose (KRBG), on ice. Adhering fat and tissue was dissected away from glands. Each gland was held in Chalazion forceps and 20–30 whole cortical sections cut with a 2 mm dia. dermatological punch. Biopsies were removed with fine forceps and one biopsy from each gland placed in each incubation flask. A total of 10 glands was processed, such that each incubation flask contained 10 biopsies (35–60 mg tissue), one from each gland. Any medullary tissue seen on the tissue core was dissected off. Light microscopy of adrenal biopsy sections showed complete cores of adrenal cortex i.e. of all 3 cortical zones, were taken. Sixty to ninety minutes elapsed between tissue collection and the commencement of incubation.

Tissue was pre-incubated for 30 min in 4 ml KRBG containing 4 g albumin‡ per 100 ml (KRBAG; which preliminary experiments had shown to be the medium for optimum production), at 37°C under an atmosphere of 95% O_2; 5% CO_2. After pre-incubation, the medium was decanted and the tissue transferred to freshly-prepared flasks containing 4 ml KRBAG, together with the stimulating agent. Incubation was allowed to proceed in a metabolic shaker (Precision

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‡ Bovine serum albumin, Cohn fraction V, Commonwealth Serum Laboratories, Melbourne, Australia.

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Scientific Co., Chicago, Ill. or Paton Industries, Adelaide, S.A.) for 2 h under the above conditions, at which time the medium was decanted and frozen until analysis. The tissue was blotted dry and weighed.

Steroid analysis. The mass of aldosterone, corticosterone, cortisol, 11-deoxycortisol, and DOC in the incubation medium was measured by double isotope derivative dilution assay[6, 7] using [^{3}H]-acetic anhydride of specific activity 80 mCi/mmol. In one time-course experiment, [^{3}H]-acetic anhydride of S.A. 150 mCi/mmol was used as esterifying agent.

The analytical method has been assessed with regard to sensitivity and precision. Specificity has been shown by constancy of ^{3}H/^{14}C ratios after multiple derivative formation and further chromatography.

Time course of steroid production. Twenty-four incubation flasks were prepared as described, and the tissue pre-incubated. Tissue was transferred to newly prepared flasks, 12 containing 4 ml KRBAG and 12 containing 4 ml KRBAG plus ACTH at 1250 mU/ml. Five, 15, 30, 60, 90 and 120 min after the commencement of incubation, 2 control and 2 ACTH-stimulated flasks were removed from the incubator and the medium immediately frozen. This experiment was performed twice.

Incubation with stimulating agents. The agents ACTH (Synacthen, Ciba), val-5-angiotensin II amide (Hypertensin, Ciba), serotonin creatinine sulphate (Nutritional Biochemicals) and potassium chloride were all dissolved in KRBAG. The response of the adrenal tissue to these agents was tested at 4 levels of agonist.

Incubation with exogenous corticosteroids. The steroids progesterone, DOC, corticosterone, and cortisol were prepared in ethanol as stock solutions of 1 mg/ml. One hundred μg of each steroid (in 0.1 ml ethanol) was added to incubation flasks, to give a final concentration of steroid of 25 μg/ml. Addition of 0.1 ml ethanol to control flasks caused no significant alteration in steroid production.

RESULTS

Unstimulated aldosterone production by 100 flasks in 28 experiments was 0.10 ± 0.06 (mean ± S.D.) μg/100 mg tissue/2 h (range, 0.02–0.25 μg/100 mg/2 h). The within-incubation variation was determined as the mean coefficient of variation for the 28 experiments, and was 15.8% for aldosterone production (Table 1). Corticosterone production was 0.03–0.32 μg/100 mg/2 h, mean production being 0.10 ± 0.06 μg/100 mg/2 h. The within-incubation coefficient of variation for corticosterone production was 13.4% Cortisol production ranged from 0.07–0.64 μg/100 mg/2 h, mean production being 0.23 ± 0.13 μg/100 mg/2 h and the within-incubation coefficient of variation 17.1%.

DOC and 11-deoxycortisol production was determined in 4 experiments. DOC production was < 10 ng/100 mg/2 h in 3 experiments and 60–70 ng/100 mg/2 h in the fourth. Unstimulated production of 11-deoxycortisol was 0.02–0.10 μg/100 mg/2 h in the 4 experiments.

Time course of steroid production. The time course of production of aldosterone and cortisol in 2 experiments is shown in Fig. 1. Aldosterone production showed positive regression, linear over the 2 h incubation period ($r = 0.973$; $P < 0.001$). Cortisol production was maximal approximately 60 min after the commencement of incubation. When ACTH (1250 mU/ml) was added to the medium, cortisol production increased and became linear (this data is not on the graph for the sake of clarity). Unstimulated corticosterone production was curvilinear, being 38%, 63%, 82% and 100% of 2 h production at 30, 60, 90 and 120 min respectively. Corticosterone production also increased and became linear after ACTH.

ACTH. The response of aldosterone, corticosterone and cortisol to ACTH in 18 experiments is shown in Fig. 2. Because of the large between-incubation variation in steroid production, all values are expressed as a percentage of the mean control production for each experiment. Aldosterone production increased significantly to a maximum of 120% of control at 12.5 mU ACTH/ml. Increasing the ACTH level to 125 or 1250 mU/ml had no further effect on aldosterone production. Corticosterone and cortisol production increased in a dose-related manner to maxima of 312% and 1229% of control respectively. In 3 experiments 11-deoxycortisol production increased in a step-wise fashion to a maximum of 310% of control production at 125 mU ACTH/ml. DOC production, in all experiments, was < 10 ng/100 mg/2 h at all doses of ACTH.

Angiotensin II. Angiotensin II caused a significant increase in aldosterone production at all concentrations tested (Fig. 3). At AII concentrations of 0.025 and 0.25 μg/ml, aldosterone production was 120% of control and increased further to 198% at 2.5 μg AII/ml ($P < 0.05$). Corticosterone production was significantly greater than control production only at 2.5 μg AII/ml ($P < 0.05$). Cortisol production was not different from control production except at 0.25 μg AII/ml when the production was 84% of mean control production ($P < 0.001$ cf control). In a single experiment ($n = 4$), DOC and 11-deoxycortisol production showed small, but significant, decreases at all concentrations of AII, except 0.025 μg/ml ($P < 0.01$ for both steroids).

Potassium. The effect of altering the medium [K] on steroid production is shown in Fig. 4. Aldosterone production was increased at all levels of [K] above 4 mmol/l, the maximum value was 179% of control at 8 mmol/l. Production then decreased in response to 12 mmol/l, although the value is still greater than control (4 mmol/l). Decreasing the [K] to 1.2 mmol/l caused aldosterone production to decrease significantly ($P < 0.001$) to 67% of mean control production. Corticosterone production also decreased in

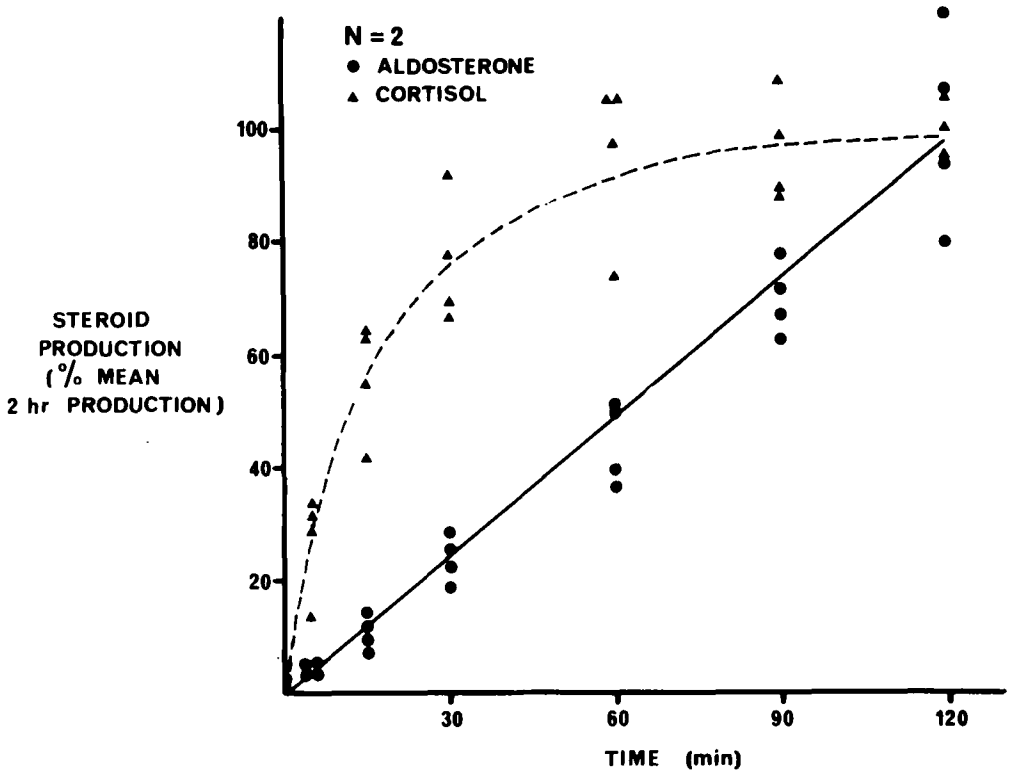

Fig. 1. Time course of unstimulated production of aldosterone (●) and cortisol (▲) by adrenal biopsies from sodium replete sheep. Number of experiments (N) = 2, number of flasks at each time interval (n) = 4.

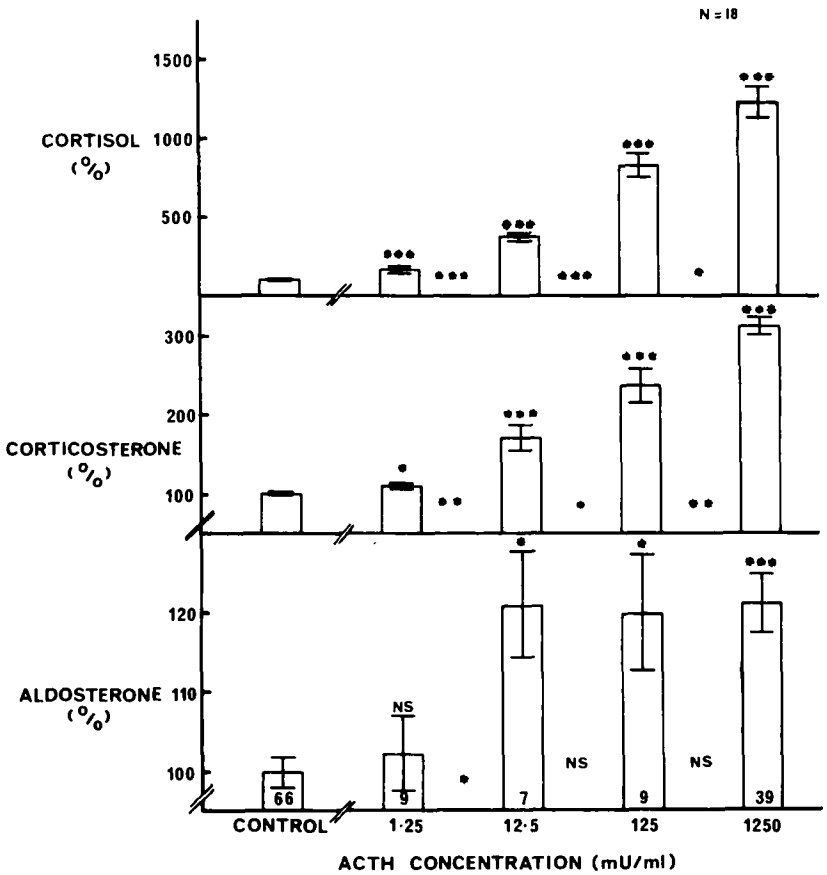

Fig. 2. The effect of ACTH on aldosterone, corticosterone and cortisol production (% of mean control production) by adrenal biopsies from sodium replete sheep. Values are given as mean ± standard error, numbers in columns = number of flasks in each group. Number of experiments (N) = 18. Values above error bars denote significance with respect to control production, and values between columns denote significance between adjacent groups (NS = not significant, * = $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.001$).

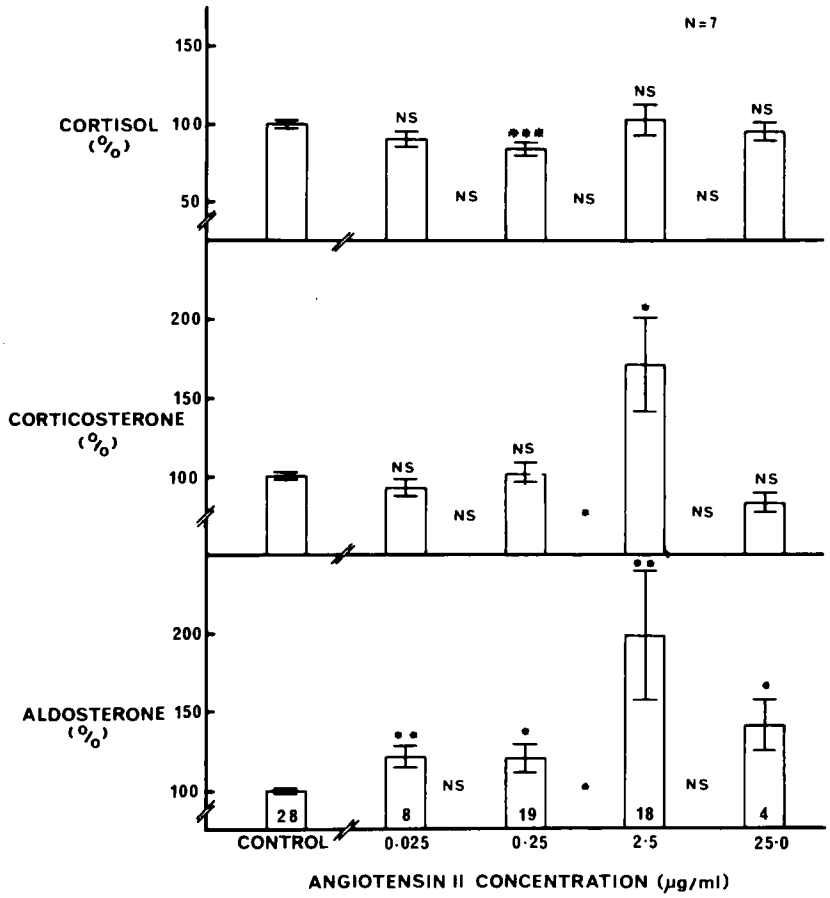

Fig. 3. The effect of angiotensin II on aldosterone, corticosterone and cortisol production (% of mean control production) by adrenal biopsies from sodium-replete sheep. See Fig. 2 for explanation of results.

response to this [K] ($P < 0.001$), and was elevated in response to increased potassium concentrations.

Cortisol production was 79% of mean control production at 1.2 mmol/l, a value significantly less than control production ($P < 0.05$). Although increasing the potassium concentration had no significant effect on cortisol production, compared to control, there is a significant difference between production at 6 mmol/l (86%) and production at 8 mmol/l (103%). In a single experiment alteration in potassium levels had no effect on 11-deoxycortisol production.

Serotonin. In one experiment ($n = 4$), serotonin at a concentration of 10^{-3} M caused aldosterone production to decrease from 0.13 ± 0.02 to 0.09 ± 0.01 μg/100 mg/2 h ($P < 0.01$). At the other concentrations tested, 10^{-4}, 10^{-5}, and 10^{-6} M serotonin had no effect on aldosterone production. Corticosterone production decreased significantly ($P < 0.05$) in response to 10^{-3} and 10^{-4} M serotonin, from 0.05 ± 0.001 to 0.04 ± 0.003 and 0.04 ± 0.003 μg/100 mg/2 h respectively, whilst 10^{-5} and 10^{-6} M serotonin had no effect. Cortisol production was increased ($P < 0.05$) from 0.12 ± 0.01 to 0.14 ± 0.01 μg/100 mg/2 h in response to 10^{-3} M serotonin, with the lower concentrations having no significant effect.

Exogenous corticosteroids. Aldosterone production was increased by incubation with substrate quantities (25 μg/ml) of progesterone, DOC and corticosterone (Table 2). Cortisol (25 μg/ml) caused aldosterone production to be significantly inhibited ($P < 0.001$). Corticosterone production was increased by the presence of 17-deoxycorticosteroid precursors. Furthermore cortisol caused a small but highly significant increase in corticosterone production ($P < 0.01$). Cortisol production was increased greatly using progesterone as precursor. DOC or corticosterone substrate also caused small yet significant increases in cortisol production.

DISCUSSION

To obtain reproducible steroid production *in vitro* it is necessary to have a homogeneous pool of tissue for sampling. Using small animals such as rat, it is possible to obtain a high degree of reproducibility of aldosterone production by the use of adrenal quarters [8]. Using large animals, however, it has

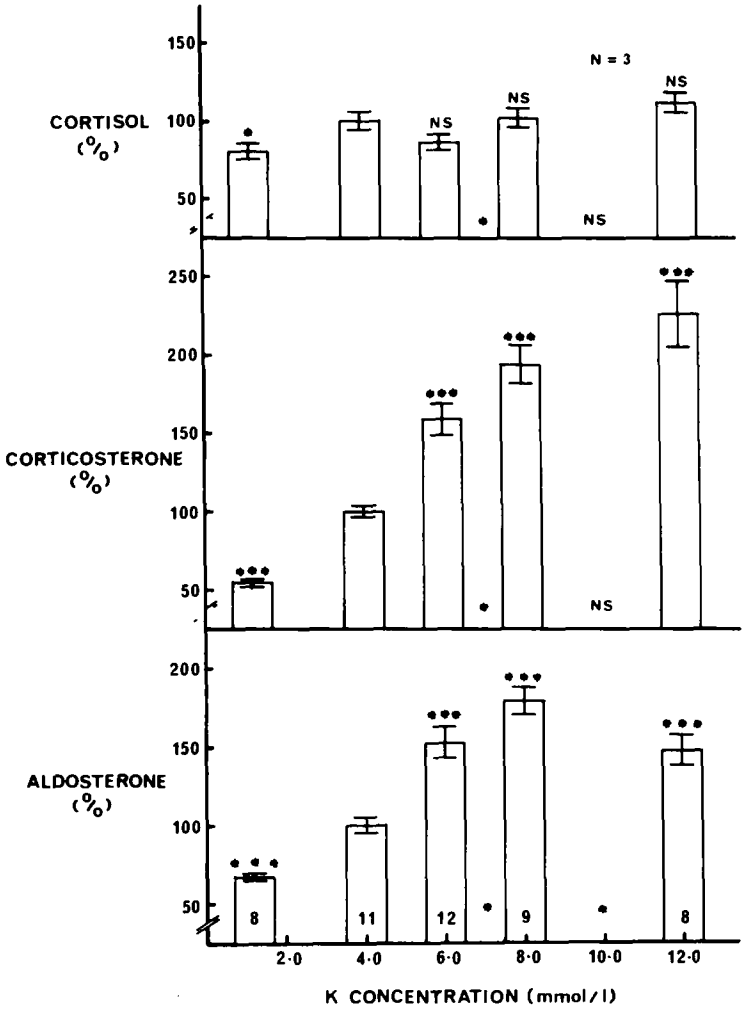

Fig. 4. The effect of potassium ions on aldosterone, corticosterone and cortisol production (% of mean control production i.e. at 4 mmol/l) by adrenal biopsies from sodium-replete sheep. See Fig. 2 for explanation of results.

been necessary to use adrenal slices [9, 10] or adrenal capsule strippings [11] in order to obtain reproducibility of production. A number of methods of tissue preparation were used during the early stages of experimentation, but in all cases, using slices or capsular strippings, the within-incubation coefficient of variation was unacceptably high (40–120%) [12]. The within-incubation coefficient of variation reported here includes intra- and inter-assay variation as well as any biological variation in production between tissue. Using the mean within-incubation coefficient of variation of aldosterone production (15.8%), it is possible to calculate that within any experiment (4 flasks per group) an alteration in

Table 1. Mean, range and within-incubation coefficient of variation (c. of v.) of steroid production by adrenal biopsies from sodium-replete sheep *in vitro*

	Mean basal production (μg/100 mg tissue/2 h) n = 100†	Range of basal steroid production (μg/100 mg/2 h) n = 100†	Within-incubation c. of v. (%) N = 28†
Aldosterone	0.10 ± 0.06*	0.02–0.25	15.8 ± 6.2%
Corticosterone	0.10 ± 0.06	0.03–0.32	13.4 ± 8.5
Cortisol	0.23 ± 0.13	0.07–0.64	17.1 ± 11.6

* Mean ± S. D.
† Number of flasks (n) = 100, number of experiments (N) = 28.

Table 2. Effect of exogenous corticosteroids (25 μg/ml) on aldosterone (ALDO), corticosterone (B) and cortisol (F) production (μg/100 mg tissue/2 h) by adrenal tissue from Na replete sheep *in vitro*

		Substrate			
	Control	Prog	DOC	B	F
ALDO	0.19 ± 0.01*	0.32 ± 0.01‡	0.40 ± 0.01‡	0.56 ± 0.02‡	0.05 ± 0.01‡
B	0.08 ± 0.01	3.96 ± 0.11‡	23.3 ± 0.99‡	—	0.13 ± 0.01†
F	0.14 ± 0.01	7.18 ± 0.26‡	0.41 ± 0.01‡	0.63 ± 0.09†	—

* Mean ± standard error. Number of flasks in each group = 4.
† $P < 0.01$ cf control.
‡ $P < 0.001$ cf control.

aldosterone production of 27.7% would be significant at $P < 0.05$.

The between-incubation variation in basal steroid production reported here (52% coefficient of variation for aldosterone) is, unfortunately, far greater than desirable. Undoubtedly this is due to differences in sodium status and the degree and time-course of stress encountered by the animals during grazing, transport to and maintenance at the abattoirs. In contrast, in a series of sodium replete sheep maintained and killed at the Institute, the between-experiment variability was of similar magnitude to the within-experiment variability [13].

Basal aldosterone production *in vitro*, 0.02–0.25 μg/100 mg/2 h, is of similar magnitude to the aldosterone secretion rate of sodium-replete sheep *in vivo* [6]. The production rates of corticosterone and cortisol *in vitro* observed over 2 h, are considerably lower than the comparable secretion rates *in vivo*. If the initial rate of cortisol production (Fig. 1) is extrapolated over a 2 h incubation, cortisol production rate is of the order 3–30 μg/h, a low value but overlapping the normal range of cortisol secretion rate by conscious undisturbed sheep [6]. Corticosterone production can be calculated, similarly, to be initially of the order 0.3–4.5 μg/h—again overlapping with the low range of normal secretion rate of corticosterone in sheep. [6].

The linearity of aldosterone production, with respect to time reported here, is in contrast to the data reported by Tait and co-workers [14, 15]. They showed a decline in aldosterone output from superfused rat adrenals which could not be explained in terms of decreased effect of exogenous ACTH or the renin/angiotensin system [15]. For steroids secreted by the zona fasciculata, the decline in steroid secretion appears to be related to the decay of effect of endogenously secreted ACTH [15, 16]. The dependence of the zona fasciculata on ACTH has been confirmed in these studies and may account for the relatively low *in vitro* production of glucocorticoids (aldosterone:corticosterone:cortisol *in vitro* was 1:1:2.3, *in vivo* the ratio was 1:15:200 [6]).

Perhaps glucocorticoid production by sheep adrenal biopsies *in vitro* is not inappropriately low, but rather, aldosterone production is inappropriately high, i.e. for the sheep zona glomerulosa the converse of Tait's "*in vivo decay*" hypothesis exists. The glomerulosa *in vitro* may be released from the influence of an *in vivo* inhibitory agent, thus causing a relative increase in aldosterone output. Evidence for the existence of an inhibitor to aldosterone *in vivo* has been presented previously [2, 3]. It is of interest to note that the decay in output of aldosterone (and 18-hydroxycorticosterone) from rat adrenal quarters was preceded by a small increase in output [15], particularly when expressed with respect to corticosterone output [14]. Although this increase may reflect an initial over-compensation of production in response to incubation, the possibility of rapid release from a specific inhibitor of aldosterone output cannot be excluded.

Basal aldosterone production by sheep adrenal biopsies *in vitro* is similar to that by ox [9] and dog adrenal sections [17, 18], greater than that observed with human adrenal slices [10], but considerably less than that observed for rat adrenal quarters [8, 19].

ACTH at concentrations of 12.5 mU/ml or greater caused a small, yet significant increase in aldosterone production by sheep adrenal biopsies, which did not appear to be dose-related. The minimum level of ACTH which is required *in vitro* for increased aldosterone production is many fold greater than that required to stimulate aldosterone secretion by sheep *in vivo*—12.5 mU/ml *in vitro* and 0.1 mU/ml (calculated) *in vivo* [20]. Similarly for cortisol and corticosterone, the levels of ACTH required for increased output *in vitro* were some 100–1000-fold in excess of the levels required *in vivo*. *In vitro* both steroids were stimulated at 1.25 mU/ml and then production increased in a dose-related manner. *In vivo* cortisol secretion from the autotransplanted sheep adrenal was stimulated by ACTH at 0.2–50 μU/ml [20–22]. Qualitatively the response of the adrenal biopsies to ACTH *in vitro* is similar to that observed in conscious undisturbed sheep *in vivo*, i.e. ACTH has a much smaller effect on aldosterone output than is shown for cortisol, and only at levels far greater than those required for the glucocorticoid [21]. The fact that higher levels of ACTH are required for aldosterone stimulation [9, 21, 23] together with the linearity of aldosterone production, suggests the receptor sites in the zona

glomerulosa show less affinity to ACTH than do those in the zona fasciculata. The overall insensitivity of the sheep adrenal biopsies to ACTH suggests the possibility that only a small percentage of the receptor sites are accessible *in vitro*.

Angiotensin II stimulated aldosterone production *in vitro* at all concentrations greater than 0.025 μg/ml. Intra-adrenal infusion of AII, in sheep, at levels greater than 0.1 μg/h stimulated aldosterone secretion [24]. This dose level is equivalent to a concentration of 0.1 ng/ml. Although AII is not necessarily the primary stimulus to aldosterone during the onset of Na depletion (reviewed in [25]), Blair-West *et al.* [1] found elevated blood levels of aldosterone at small sodium deficits, associated with blood AII levels of 0.05 ng/ml.

The disparity between the levels of AII required to stimulate aldosterone *in vitro* and *in vivo* may again suggest that only a small percentage of receptor sites are accessible for hormone interaction. Alternatively, the relative insensitivity to AII *in vitro* may be because the 2–8 heptapeptide of AII (angiotensin III, AIII) is the physiological stimulus and there is a relative inability of the tissue to perform the necessary amino acid cleavage. AIII is a potent stimulus to aldosterone both *in vivo* [26] and *in vitro* [27–29], and data from Chiu and Peach [30] suggested that the adrenal angiotensin receptor showed greater affinity for AIII than for AII.

As with ACTH the qualitative effects of AII are similar *in vivo* and *in vitro*, i.e. AII had little or no effect on glucocorticoid secretion *in vivo* in sheep [24] and dog [31], and only at levels of agonist far in excess of those required to stimulate aldosterone [31].

The *in vitro* data presented here is further evidence that AII acts predominantly on the zona glomerulosa. Angiotensin stimulates aldosterone production or secretion but not cortisol in the sheep both *in vitro* and *in vivo* [24]. Similarly in the rat *in vitro*, Müller [32] observed high levels of angiotensin stimulated aldosterone, corticosterone and DOC production by "capsular" adrenals but had no effect on steroid production by "decapsulated" adrenal tissue. Kaplan and Bartter [9, 33] using beef adrenal tissue, observed, however, that AII stimulated both cortisol and corticosterone production.

Potassium ions are an extremely potent stimulus to aldosterone production, increased outputs being observed in response to an increase of 2 mmol/l, although an increase of 0.5 mmol/l would be expected to cause significant stimulation. Increments in plasma [K] of 0.5 mmol/l consistently increased aldosterone secretion from the sheep adrenal autotransplant, with smaller increments being effective in a majority of experiments [34].

The direct effect of K on the adrenal appears to be limited to the zona glomerulosa. In the rat, K had no effect on corticosterone production by quartered adrenals [8] or "decapsulated" tissue [32]. K ions stimulated the output of both aldosterone and corticosterone from beef adrenal slices, but did not affect cortisol [9]. In contrast, cortisol production was also stimulated by K in dog adrenal slices [18]. The differences between K levels required for aldosterone stimulation *in vivo* and *in vitro* are much smaller than are the differences for the other two stimuli. This is probably due to the fact that the cells are far more accessible to the lower molecular weight K ions than they are to the larger polypeptide hormones. Alternatively the disparity may be because K ions act directly on the adrenal, while peptides particularly AII, may require some molecular modification before they are fully active.

Serotonin at high concentrations (10^{-3} mol/l) caused a slight decrease in aldosterone and corticosterone production and a small increase in cortisol production by sheep adrenal biopsies *in vitro*. It had no effect on steroid secretion when infused into the sheep adrenal autotransplant [20]. Serotonin is an extremely potent stimulus to aldosterone production by rat adrenal tissue *in vitro* [35, 36] although the physiological role of serotonin on aldosterone production is unknown [37].

The effect of precursor steroids on aldosterone production supports the concept that progesterone, DOC and corticosterone are intermediate compounds in a biosynthetic pathway to aldosterone in the sheep. Incubation of rat and beef adrenal tissue with similar concentrations of these 3 steroids also increased aldosterone output, although in contrast all had the same quantitative effect [8, 33]. The inhibition of aldosterone production by high concentrations of cortisol has been reported also for rat adrenal quarters *in vitro* [38].

The effect of exogenous steroids on corticosterone production is far greater than that observed in rat adrenal quarters [8] where again the effects of DOC and progesterone were similar. The slight increase in corticosterone production in response to cortisol reported here for sheep may be due to an accumulation of corticosterone caused by some cortisol-induced late-site enzyme inhibition [38, 39].

It has been suggested that cortisol production is predominantly *via* 17 α-hydroxyprogesterone, bypassing progesterone [40]. The data presented in Table 2 although not distinguishing between pathways suggests that progesterone, *in vitro*, is a good precursor to cortisol. The observation that DOC and corticosterone can be converted to cortisol is of interest and is probably due to direct 17 α-hydroxylation of the former steroids. The physiological significance of such a biosynthetic mechanism however is unknown.

In summary, a novel method for the preparation of adrenal tissue from large species (sheep) has been described. The method is simple and up to 30 separate incubation flasks can be prepared for any incubation, with a within-incubation c. of v. of 16% for aldosterone production. Because of the characteristics of production the method could be of great use as a bioassay system for the testing of various biological

extracts for aldosterone-stimulating or -inhibiting activity and also for further study into the biosynthetic pathways to and mechanisms of control of aldosterone.

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