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EFFECT OF ADRENOCORTICOTROPHIC HORMONE ON THE *IN VIVO* OUTPUT OF ADENOSINE 3′5′ CYCLIC MONOPHOSPHATE AND CORTICOSTEROIDS BY THE SHEEP ADRENAL GLAND

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SUMMARY

The effect of intravenous infusion of adrenocorticotrophic hormone (ACTH) (12 u/h) on the adrenal venous concentration of adenosine 3′5′ cyclic monophosphate (c-AMP) was studied in five conscious sheep with a cervical adrenal autotransplant. In addition adrenal vein blood was assayed for cortisol, corticosterone and aldosterone.

ACTH infusion caused a rapid increase in adrenal c-AMP output which was apparent within the first minute of infusion and which appeared to precede the increase in steroid production.

Peak c-AMP output was 10–100 fold greater than pre-infusion output and was reached within 15 min of infusion. Maximum steroid production also occurred at about 15 min. However, in contrast to adrenal steroid production, the increase in adrenal c-AMP output was not sustained at the maximum rate and decreased gradually during the infusion period. In sheep with maximum pre-infusion steroid production, adrenal c-AMP output was increased by ACTH infusion.

The basal c-AMP concentration in arterial plasma was 10–35 nM and no significant increase was observed during ACTH infusion.

The response pattern of c-AMP and steroid secretion by the adrenal gland *in vivo* to a large dose of ACTH confirms that previously reported from *in vitro* studies.

INTRODUCTION

Adenosine 3′5′ cyclic monophosphate (c-AMP) has been implicated in the steroidogenic response of the adrenal glands to the adrenocorticotrophic hormone (ACTH) since Haynes [1] showed that ACTH specifically increased the c-AMP content of incubated bovine adrenal slices. Subsequently, Haynes, Koritz and Peron [2] and Roberts, Creange and Young [3] demonstrated that c-AMP alone would stimulate steroidogenesis in those rat adrenal slices and homogenates which would also respond to ACTH. Grahame-Smith, Butcher, Ney and Sutherland [4] using the rat adrenal gland, found that ACTH stimulated the production of c-AMP in whole tissue incubated *in vivo* and *in vitro* and also in tissue homogenates.

Using the isolated cat adrenal gland perfused *in situ* Jaanus, Rosenstein and Rubin [5] showed an increased secretion of corticosteroids into the adrenal venous system and an increase in the adrenal tissue c-AMP concentration in response to low concentrations of ACTH [6]. Peytremann, Nicholson, Hardman and Liddle [7] demonstrated that both corticosterone and c-AMP were secreted into the rat adrenal venous blood in response to ACTH stimulation.

These data suggest a relationship between ACTH stimulated steroidogenesis, an increase in tissue c-AMP concentration and its release into the extracellular space. In the present study, an *in vivo* model was used. Trained conscious sheep with cervical adrenal autotransplants were given a constant i.v. infusion of ACTH. Adrenal venous blood samples were collected for the measurement of adrenal c-AMP and corticosteroid output.

EXPERIMENTAL

Animals. Five conscious wethers (body weight 40–50 kg) with a left adrenal autotransplant, exteriorised in a jugulo-carotid skin loop [8, 9] were used. The transplantation together with right adrenalectomy and exteriorisation of the contralateral carotid artery had been performed several years previously.

Experimental protocol. The studies were commenced between 1100 and 1300—at least 2 h after cannulation of the right and left jugular veins. After collecting basal blood samples the animals received a constant intravenous infusion of β1–24 ACTH (Synacthen, Ciba–Geigy) into the right jugular vein at a rate of 12 I.U./h ($\equiv$ 120 μg/h) for either 30 min (5 experiments) or 60 min (3 experiments). This dose of ACTH was known to produce a blood concentration greater than that required for maximal steroid production [10] and was used to avoid the biphasic steroid response obtained with submaximal adrenal stimulation [11].

During the experiment, the jugular vein and carotid artery were occluded, distal to the gland, by an inflated rubber cuff. Timed volume, adrenal venous

blood samples were collected by occluding the left jugular vein proximal to the gland. The blood was siphoned through the left jugular vein cannula into heparinised containers stored on ice. In the four experiments, samples were collected each min for the 12 min following the commencement of the ACTH infusion and during this time there was no recirculation of the adrenal venous output. In three animals, arterial blood samples were taken by arterial puncture of the right carotid artery during and after the ACTH infusion and in three others, only basal arterial and adrenal venous samples were obtained.

The blood samples for c-AMP measurement were centrifuged at 2000 *g* for 15 min at 4°C and the separated plasma diluted (1:1 v/v) with 8 mM theophylline in 50 nM Tris–HCl at pH 7·4. These samples were stored at 0°C until assayed, which was within 60 min after completing the experiment. The above procedure had been shown to produce no loss of c-AMP.

Measurement of c-AMP. The c-AMP concentration of plasma and whole blood was measured using a competitive protein binding assay, similar to that described by Brown, Albano, Ekins, Sgherzi and Tampion [12]. The binding protein was prepared from bovine adrenal cortical tissue. The assay incubation contained 250 μg of binding protein, as determined by the Folin phenol reagent method [13] using bovine albumin as a standard. The protein was in 300 μl of 50 mM Tris–HCl pH 7·4, 8 mM theophylline, 6 mM 2-mercaptoethanol and 25 nCi [^{3}H]-c-AMP (The Radiochemical Centre, Amersham; S.A. 27·5 Ci/mmol) was used as the tracer. A standard response curve was prepared over the range 0–8 pmol of c-AMP per incubation, and the unknowns were assayed at two appropriate dilutions on the sensitive part of the curve. After an incubation period of 120 min at 4°C, the free [^{3}H]-c-AMP was adsorbed with 7·5 mg charcoal (Norit A) in 1·5% BSA (fraction V, Commonwealth Serum Laboratories) in 500 μl 50 mM Tris–HCl pH 7·4. The assay tubes were rapidly mixed before centrifuging at 2000 *g* for 15 min at 4°C. A 500 μl aliquot of the supernatant was added to 10 ml of a scintillation cocktail (250 ml Triton X-100, 5·36 g PPO, 0·536 g POPOP, 750 ml Toluene) and counted in a Packard Tricarb Liquid Scintillation Spectrometer (Model 3330) for 5 min (10,000 c.p.m.) with a counting efficiency for ^{3}H of 25%. The assay precision was ± 0·1 pmol at 1 pmol and the coefficient of variation between assays was 11%. In checks on the assay specificity, the only cross-reactivity demonstrated was with guanosine 3′5′ cyclic monophosphate at a concentration greater than 500 pmol per incubation. The plasma dilution was usually assayed without extraction. Some plasma and whole blood samples were deproteinated using perchloric acid. Following centrifugation, an aliquot of the supernatant was adjusted to pH 7·4 using K_2CO_3 and the salt was precipitated by centrifugation at 0°C. An aliquot of this supernatant was assayed. The extraction recovery was monitored using [^{3}H]-c-AMP (2·5 μCi/ml plasma or whole blood) and was found to be about 80%. After correction for extraction losses there was little difference between the whole blood, plasma and unextracted plasma c-AMP concentrations (Table 1). Other plasmas were deproteinated by boiling [14] and serial dilutions of both the plasma and deproteinated extract assayed and found to be linear, passing through the origin. These samples were also assayed using a commercial radioimmunoassay (Collaborative Research) and good agreement was found to exist between the sample values obtained from both assays (Table 2). Incubation of plasma containing 16·4 nM c-AMP with 3′5′ cyclic nucleotide phosphodiesterase (Sigma) for 30 min at 37°C resulted in complete loss of measure c-AMP activity (Table 2). Samples stored at −20°C in the presence of 4 mM theophylline were stable for 48 h, however, longer storage periods resulted in a gradual loss of measured c-AMP.

Measurement of steroid hormones. Cortisol, corticosterone and aldosterone concentrations in adrenal venous blood samples were measured by the double isotope derivative dilution method of Coghlan, Wintour and Scoggins [15].

RESULTS

Basal secretion by the adrenal gland. Basal secretion by the adrenal gland of c-AMP, cortisol, corticosterone and aldosterone measured in 8 experiments using 6 sheep is shown in Table 3 at times 0 min. The

Table 1. Comparison of the whole blood and plasma c-AMP concentration in sheep. The plasma and whole blood were deproteinated before being assayed. The assay values were corrected for losses using 2·5 nCi [^{3}H] c-AMP/ml of plasma or whole blood as a recovery marker. There was no significant ($P > 0·05$) difference between whole blood and plasma concentrations

	c-AMP Concentration, nM		
Sheep	Whole blood	Plasma	Unextracted plasma
No. 1	40	35	36
No. 2	41	41	38
No. 3	37	36	36
No. 4	50	39	—
No. 5	48	34	—

Table 2. c-AMP concentration (nM) in 6 sheep plasma samples assayed directly and after extraction of the deproteinated sample by either protein binding assay or radioimmunoassay. The effect of 3'5' cyclic nucleotide phosphodiesterase on a sample containing 16·4 nM c-AMP is also shown

Sample	Protein binding assay		Radioimmunoassay
	Plasma	Deproteinated extract	Deproteinated extract
1	395	400	407
2	240	199	281
3	87	78	132
4	77	63	53
5	43	40	40
6*	< 0·1	<0·1	<0·1

* After incubation with 3'5' cyclic nucleotide phosphodiesterase

Table 3. The relationship between c-AMP and cortisol, corticosterone and aldosterone output in adrenal venous blood during systemic infusion of ACTH at a rate of 12 IU/h

Sheep	Time min	c-AMP nmol/min	Cortisol nmol/min	Corticosterone, nmol/min	Aldosterone, nmol/min
	0	0·031	0·35	0·02	0·003
	12	1·06	59·0	5·19	0·03
	25	0·54	62·1	5·19	0·23
Waldo 1	35	0·41	56·2	4·91	0·20
	45	0·32	60·7	4·91	0·18
	55	0·21	69·8	5·63	0·20
	Post 20	0·12	63·5	5·00	0·21
	0	0·087	16·2	0·64	0·01
	3	0·74	41·6	2·13	0·21
	9	1·41	52·1	3·98	0·28
Waldo 2	17	1.34	57·9	3·66	0·28
	25	1·46	52·7	3·40	0·14
	Post 20	0·78	54·6	4·04	0·14
	45	0·09	6·4	0·31	0·06
	0	0·055	2·4	0·13	0·002
	15	5·36	77·8	8·80	0·29
	25	4·75	87·5	10·10	0·36
Ham	35	4·47	85·5	10·10	0·31
	45	1·83	79·4	10·40	0·28
	55	3·70	82·8	10·70	0·30
	Post 20	3·60	5·8	2·00	0·01
	0	0·11	0·18	0·05	0·05
	6	1·17	63·2	3·31	0·25
Seamus	14	0·97	60·9	3·35	0·19
	24	1·14	66·8	3·35	0·11
	Post 20	1·00	64·8	3·00	0·04
	0	0·28	8·0	0·35	0·01
	6	0·36	37·2	2·97	0·16
Giovanni	14	0·61	37·2	3·26	0·17
	19	0·79	36·4	3·17	0·15
	Post 20	0·58	41·3	3·32	0·11
	0	0·40	75·6	5·57	0·25
	15	6·71	71·9	8·54	0·28
Massey 1	20	2·39	107·0	11·37	0·26
	35	2·45	101·4	12·32	0·26
	45	1·92	75·4	8·89	0·19
	Post 30	2·13	158·4	12·90	0·26
	0	0·45	61·8	4·56	0·04
	4	3·10	47·4	2·74	0·06
Massey 2	9	4·00	67·6	7·79	0·25
	17	2·65	83·6	9·46	0·22
	25	3·24	88·8	8·91	0·15
	Post 20	4·70	71·4	3·49	0·09
	0	0·55	72·9	7·45	0·15
	15	6·38	56·7	6·49	0·37
Amos	20	2·47	58·7	6·40	0·30
	25	1·61	29·2	3·03	0·11
	35	2·28	41·5	4·00	0·17

adrenal venous plasma c-AMP concentration ranged from 5–30 nM. The adrenal blood secretion rate was calculated from the measured adrenal blood flow and the plasma concentration. This can be done since it has been established that the plasma and whole blood concentrations of c-AMP are similar in the sheep (Table 1). There was a positive correlation between adrenal c-AMP and steroid secretion. In three experiments, (Massey 1, 2 and Amos) elevated steroid production was associated with increased c-AMP secretion.

In 7 experiments, both arterial and venous c-AMP plasma concentrations were measured. There was no significant difference between the two samples from the same animal, or between the mean concentrations (arterial = 21·9 nM: venous = 20·1 nM). In two sheep, the jugular venous plasma concentrations were not different from those found in the adrenal vein (24·7 nM and 23·4 nM respectively).

Effect of ACTH. The effect of ACTH upon c-AMP secretion by the adrenal gland in 4 sheep is shown in Fig. 1. The increase in c-AMP secretion was rapid and apparent within the first min. In one experiment (Waldo 1), the peak c-AMP secretion was considerably less than in the other three, although the pre-infusion secretion was not significantly different from that of Ham and both were less than those of Massey 1 and Amos.

The changes in adrenal c-AMP and steroid secretion during ACTH administration are summarised in Table 3. In 3 experiments (Waldo 2, Seamus and Giovanni) increased steroid production was apparent at the earliest sampling and was sustained during the ACTH infusion. In another experiment (Massey 2) the adrenal gland, for reasons other than the experimental protocol, was being maximally stimulated before commencing the ACTH infusion, and no further increase in steroid production was elicited by ACTH. However, a 10-fold increase in c-AMP secretion occurred within 10 min, indicating a significant capacity for c-AMP production in the presence of maximum steroid production. A similar pattern appeared to exist in 2 other experiments (Massey 2 and Amos), where a rapid increase in c-AMP secretion occurred despite a pre-infusion cortisol production of greater than 62 nM/min (Table 3).

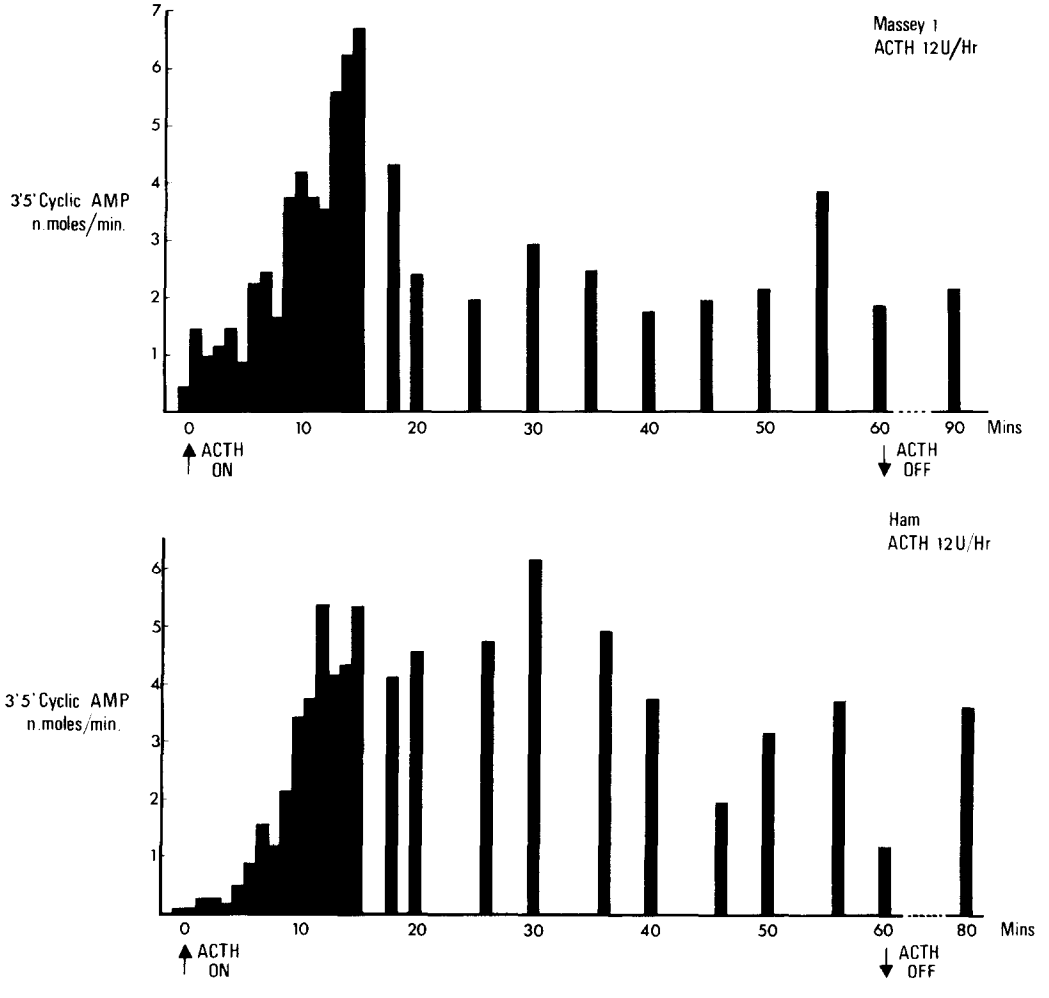

Fig. 1. The effect of intravenous ACTH infusion on secretion of c-AMP by the sheep adrenal gland.

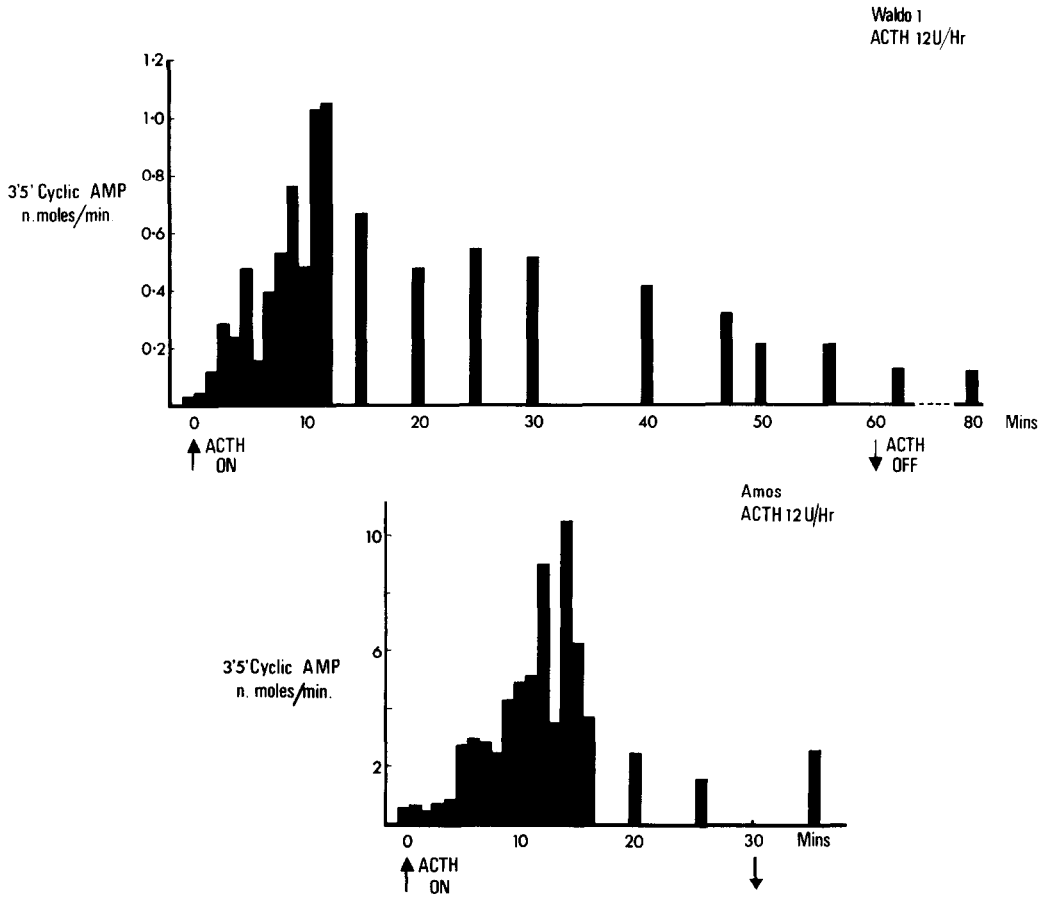

Fig. 1. *cont.*

In 2 experiments, Seamus and Massey 2, there are changes in aldosterone secretion which do not parallel changes in cortisol production. These differences are probably due to the different response characteristics of the zona fasciculata and zona glomerulosa to ACTH.

In the 3 experiments in which the ACTH infusion was made for 60 min, the peak adrenal c-AMP secretion was not sustained throughout the infusion period, although it remained greater than the pre-infusion secretion (Fig. 2). Cortisol production and also that of corticosterone and aldosterone, was maintained at stimulated levels throughout the infusion, even though c-AMP secretion had declined. Output of both c-AMP and cortisol was still elevated above pre-infusion levels 20 min after the cessation of the ACTH infusion.

Table 4 shows that in 3 experiments an increase in the arterial plasma c-AMP concentration occurred during the ACTH infusion. This increase was small and not significant when compared with the large increase measured in the adrenal venous plasma c-AMP concentration.

DISCUSSION

This *in vivo* study in the conscious sheep has demonstrated that ACTH stimulation of the adrenal gland is associated with a rapid increase in the production and release of c-AMP into the adrenal vein blood.

Secretion of c-AMP by the adrenal and its association with steroid release has been previously shown in the cat [16], rat [7], dog [17] and sheep [18]. ACTH stimulation causes an increase in the release of both c-AMP and steroids into adrenal vein blood [5, 6, 7, 17, 18] although there would appear to be some dissociation which will be discussed later.

The dynamics of the steroidogenic response of the adrenal gland to ACTH stimulation is well documented in beef [19, 20], rat [21], sheep [22, 23], dog [24] and cat [5]. The steroidogenic response has been shown to be dependent upon the ACTH dose used [22]. With maximum ACTH stimulation, the increase in steroidogenesis is apparent at 3–4 min [22], as also seen in the present study, and once established remains constant for the duration of the stimulation.

In all adrenal systems studied either *in vivo* or *in vitro*, the increased production of c-AMP in response to ACTH precedes the steroidogenic response [4, 18]. In this *in vivo* study using the whole gland, the increased secretion of c-AMP was measured at one min and would have preceded any increase in steroid production [23].

The delay between increased c-AMP production and steroid production is believed to be due to poor

Table 4. Arterial and adrenal venous plasma c-AMP concentrations (nM) prior to, during and after ACTH infusion in 3 sheep

		Pre-ACTH	ACTH-30 min	Post-ACTH
Seamus	Arterial	11·4	17·0	19·0
	Venous	9·8	58·0	53·4
Giovanni	Arterial	12·5	19·8	9·8
	Venous	15·5	39·6	29·2
Massey	Arterial	30·0	23·8	—
	Venous	28·0	>300	—

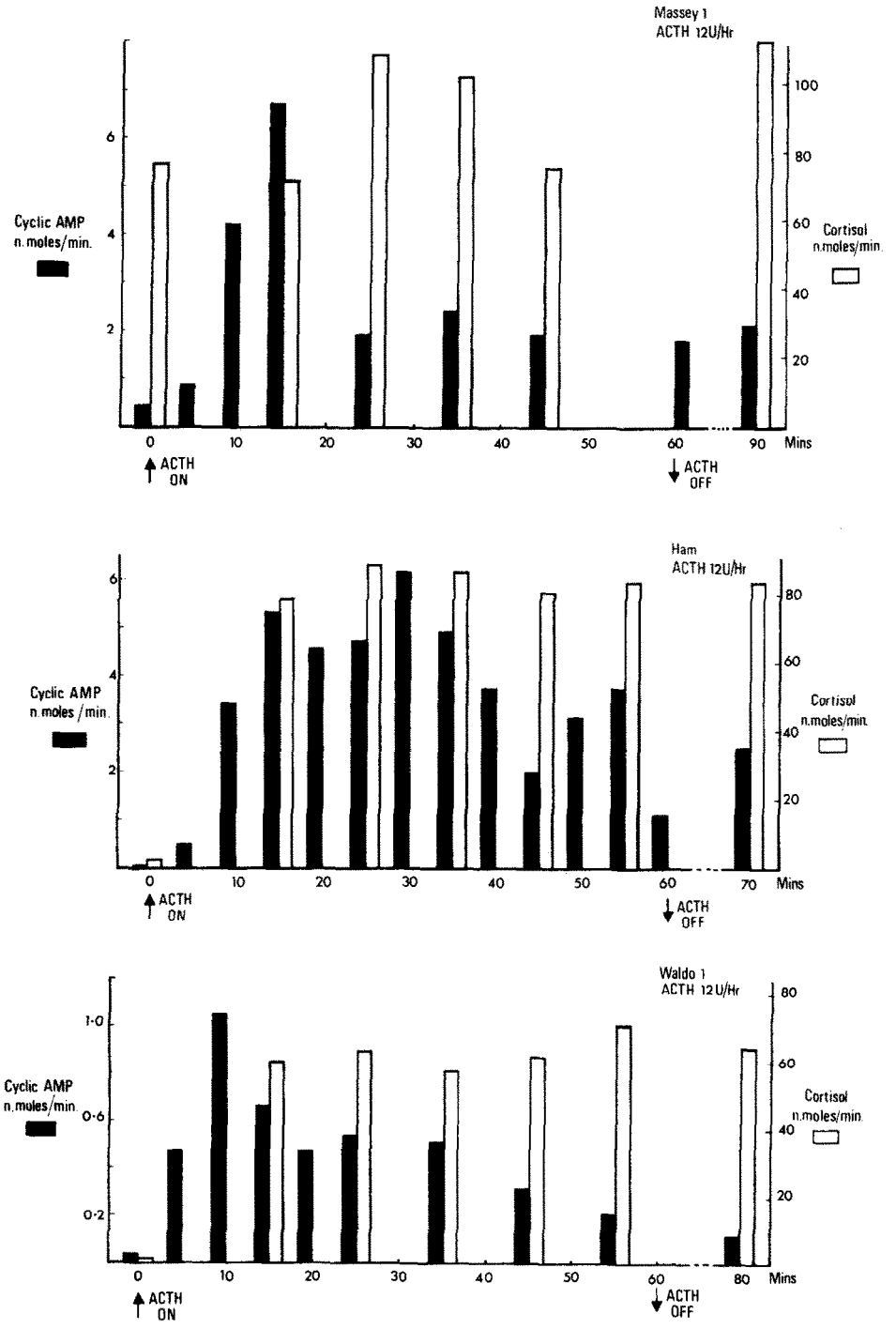

Fig. 2

penetration of the hormone into the adrenal tissue or to delay in the accumulation of sufficient c-AMP to produce the intracellular effect. The same time-lag had been demonstrated *in vitro* using isolated rat adrenal cell suspensions, where presumbly penetration was not such a problem [25]. In addition Richardson and Schulster [26] showed a time-lag of 3 min in adrenal cell suspensions using either c-AMP or ACTH diazotised to polyacrylamide beads. Thus ACTH can stimulate steroidogenesis without entering the adrenal cell. The delay would appear to be due to the translation betwen c-AMP production and steroidogenesis through a protein synthesis process [27]. If this synthesis is blocked e.g. by cycloheximide [4, 28], c-AMP production can still be stimulated by ACTH and the c-AMP-dependent protein kinase activity is not affected [29]. Such a protein synthesis process would be consistent with the 3 min delay before steroidogenesis [26].

In the present study, ACTH could stimulate adrenal c-AMP secretion in the presence of maximum steroid secretion. The two sheep with high pre-infusion steroid production failed to produce a further increase in steroid production during ACTH infusion but did show a marked increase in c-AMP secretion. A similar phenomenon has been shown by Mackie *et al.* [25] using isolated adrenal cells, in which the concentration of ACTH, which just elicited maximum steroidogenesis, resulted in an increase in c-AMP production which was 12% of the peak production achieved with large doses of ACTH. Other similar observations have been reported by Grahame-Smith *et al.* [4], Nakamure, Ide, Okabayaski and Tanaka [30], Seelig and Sayers [31], Peytremann *et al.* [7], Espiner *et al.* [18] and Bennett, Bullock, Lowry, McMartin and Peters [32] who suggested that maximum stimulation of steroidogenesis occurred at an ACTH concentration which did not result in fully occupied receptors.

Adrenal c-AMP secretion was not sustained in the presence of a continued high ACTH concentration as shown in the current experiments. Other reports of *in vitro* and *in vivo* experiments demonstrate similar declines in c-AMP production, after rapid initial increases, with constant ACTH stimulation [33, 18].

This decline in c-AMP production and secretion could be related to a refractoriness or decrease in receptor sensitivity or an increase in the clearance of c-AMP within the gland. Tauton, Roth and Pastan [34] found that ACTH stimulated the production of c-AMP but not its breakdown in broken cell preparations and in other *in vitro* and *in vivo* studies it has been shown that ACTH may be bound in the whole gland [35–38]. Thus refractoriness within the receptor-adenyl cyclase system may explain the decrease in c-AMP production with sustained maximal ACTH stimulation of the adrenal cell.

In conclusion, the data presented from this study of the whole adrenal gland in the conscious animal confirms data previously published using other *in vivo* systems and would be consistent with a second messenger role for c-AMP in the pathway between ACTH and steroid production.

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