THE RESPONSE OF CIRCULATING CORTISOL LEVELS IN SHEEP TO VARIOUS STRESSES AND TO RESERPINE ADMINISTRATION

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Abstract

The effects of several stresses on circulating cortisol levels in sheep were investigated. The aim was to devise a means of assessing the effects of certain treatments on the response of cortisol to stress. Short periods of fasting proved effective stresses initially but routine applications of this stress resulted in greatly diminished cortisol responses. This change in response was shown to be to some extent reversible. Longer fasts produced very variable changes in cortisol levels, but consistently elevated free fatty acid levels, while shearing and short periods of transportation produced consistent elevations in cortisol levels but variable changes in free fatty acid concentrations. Levels of cortisol following transportation and shearing approximated to those in lambs at slaughter under commercial conditions. The daily administration of 3, 5, or 7 μg of reserpine per kilogram body weight for 3 days did not affect basal circulating cortisol levels or the degree of response of these levels to stress.

I. INTRODUCTION

Since the productivity of domestic animals may be determined to some extent by the activity of the adrenal cortex (Bassett 1963; Carroll *et al.* 1963; Gavora and Kondra 1970; Purchas *et al.* 1971), it is appropriate that means of manipulating this activity be investigated. In normal animals cortisol and corticosterone production appears to be largely determined by the environmental stresses to which an animal is exposed, with these stresses exerting their effect through hypothalamic stimulation, corticotrophin releasing factor (CRF) production, and adrenocorticotropic hormone (ACTH) production in that sequence (Mangili *et al.* 1966; Dallman and Yates 1969). The demonstration by Purchas *et al.* (1971) of negative relationships between circulating levels of corticosteroids at slaughter and growth rate or meat tenderness of cattle, therefore, suggests that the more rapidly growing animals, for example, were less responsive to environmental stresses at some point in the hypothalamic–pituitary–adrenal axis. The results, however, do not indicate how this apparent lower responsiveness to stress influenced growth rates and tenderness.

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It should be possible to obtain some information on this by inhibiting the hypothalamic–pituitary–adrenal axis at different points and testing for effects on growth or meat tenderness. Inhibition of the pathway at the adrenal level was considered undesirable since low meat quality has been associated with deficiencies in adrenal steroid production in the pig (Judge *et al.* 1968).

The pharmacology of agents which appear to inhibit the secretion of ACTH has been reviewed by Munson (1963). He concluded that a reliable inhibitor of ACTH secretion without complicating side effects remained to be discovered. In the case of reserpine, for example, he noted that the situation was not clear, although there did appear to be some inhibiting effect on the response of ACTH secretion to stress.

This paper reports the results of a series of experiments concerned with the effects of stresses and reserpine administration on circulating cortisol levels in sheep. Free fatty acid levels were measured in some experiments as a second index of stress. Initially, a standard short fasting period was an effective stress but repeated use of this technique appeared to result in a decreased response. Further experiments were conducted to investigate this phenomenon and the effect of other stresses including longer fasts, shearing, weighing, transportation, and restraint.

II. Materials and Methods

(*a*) *Animals*

Six mature Border Leicester × Dorset Horn wethers were used in all experiments except one, in which 24 lambs were used. All animals were penned indoors individually on slats. The wethers were fed a pelleted ration comprising 60% barley, 25% lucerne chaff, 10% oaten chaff, 2% urea, and 1% each of NaCl, $CaCO_3$, and $CaSO_4$. Vitamins D, E, and A were administered orally at intervals of approximately 4 weeks. The lambs received lucerne chaff *ad libitum* and all animals had free access to water.

(*b*) *Experimental Methods*

Reserpine (marketed as Serpasil by Ciba-Geigy Aust. Ltd.) was diluted in propylene glycol and then in an equal volume of citrate buffer (0·02M citrate, 0·81% NaCl, pH 3·0). It was administered intramuscularly.

Blood samples were taken from the jugular vein by venipuncture. After standing at room temperature for approximately 1 hr and at 0–4°C for at least 4 hr, serum was poured off and centrifuged at 3000 *g* for 20 min. Serum levels of free fatty acids (FFA) were estimated by the method of Itaya and Ui (1965).

Corticosteroids were assayed by the method of Bassett and Hinks (1969) with the following two minor modifications. First, in the preparation of the corticosteroid binding globulin (CBG) reagent, endogenous corticosteroids were removed from the dog plasma by the use of Florisil rather than by gel-filtration with Sephadex. 75 ml of 5% dog plasma in borate buffer (0·1M, pH 7·6) were shaken for 15 min in a water-bath at 45°C with 4 g of Florisil (60–100 mesh). Prior to use, fines were removed from the Florisil by twice decanting with distilled water. After incubation, the solution was separated from the Florisil by filtration through a sintered-glass funnel at 45°C. The resulting 5% CBG solution was frozen in 15-ml aliquots and diluted to a 2% solution on the day of use. Secondly, the 0·1-ml ethanol extracts were evaporated by incubating the tubes (3 by 3/8 in.) in an oven at 43°C for 12 hr rather than by using a stream of nitrogen.

Throughout this paper the results of the competitive protein-binding assay are expressed in terms of cortisol concentrations, although it is recognized that other corticosteroids are involved (Bassett and Hinks 1969) and possibly other compounds also (Willett and Erb 1972).

III. Results

(*a*) *Fasting Effects*

The effect of an 18–20-hr fast on circulating cortisol levels in sheep which were accustomed to receiving food *ad libitum* is shown in Figure 1,*A*. It appeared from these results that such a fast was an effective stress in terms of cortisol production, and so it was used routinely in an experiment over an 8-week period. At the end of this period the effectiveness of this stress was checked and the results, shown in Figure 1,*B*, indicated that cortisol levels were no longer elevated after the fast. This

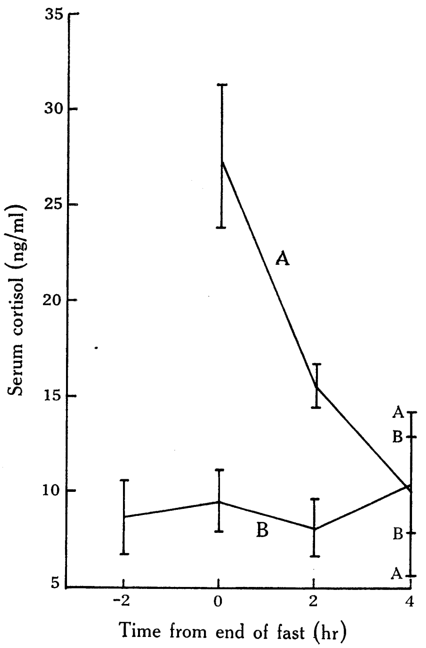

Fig. 1.—Changes in circulating cortisol levels in sheep following an 18 to 20-hr fast. *A*, sheep unaccustomed to the fast. *B*, sheep routinely fasted for 18–20 hr per day. Values are means ±S.E. Sheep fed at 0 hr.

apparent loss of response could have been tested by submitting different animals to the same treatment and testing for the same effect. Rather than this, however, the reversibility of the effect was tested using the same animals. The change from being fed for only 4–6 hr per day (to which they were accustomed) to being fed *ad libitum* was made gradually by increasing the feed offered by 300-g increments each week. Thus, the feed offered per animal per day was increased from 300 g during the first week to 2100 g during the seventh. The animals had free access to the feed containers at all times except on day 6 of each week when the feed was removed between 4 and 5 hr after it had been offered.

The mean intakes as the quantity offered was increased are shown in Table 1, for the days when the feed was left in front of the animals and for the day when it was removed after 4–5 hr. The difference between the two values, which should be proportional to the difference between fasting time on day 6 and on the other days, increased as the amount offered increased above 900 g per day [Fig. 2(*a*)]. Figures

2(*b*) and 2(*c*) show the changes in cortisol and FFA concentrations from before feeding on day 7 to 100 min after feeding. Levels at 200 min after feeding were similar to those at 100 min. Regression analysis revealed a significant positive linear relationship between feed offered per day and decrease in cortisol levels from before feeding to 100 min after feeding as the feed offered increased from 900 to 2100 g per day.

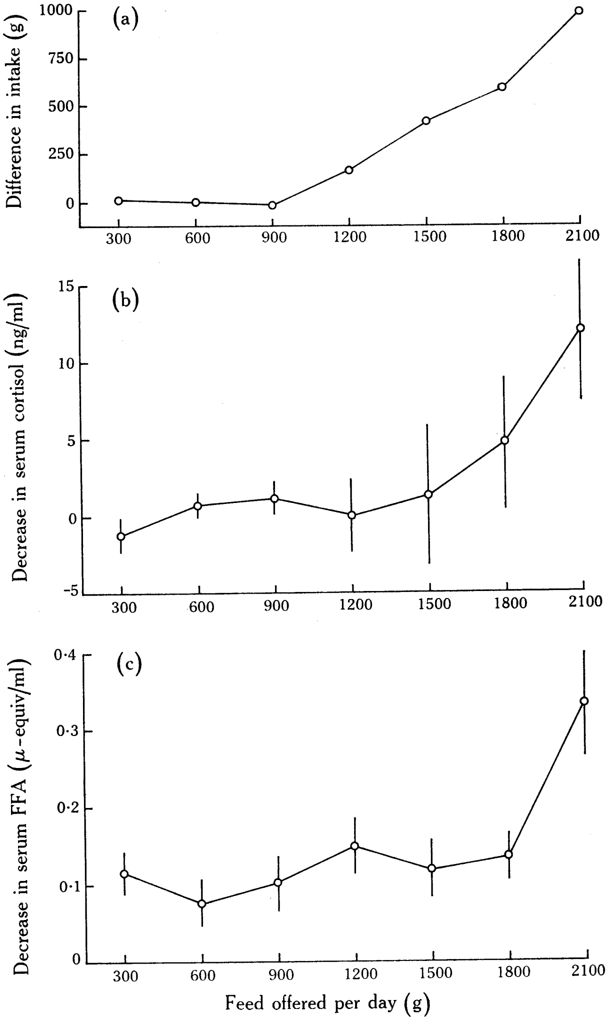

Fig. 2.—Effect of quantity of feed offered to sheep per day on: (*a*) the difference between quantity consumed in 4–6 hr on day 6 and that consumed in 24 hr on other days; (*b*) the decrease in serum cortisol levels from the end of an 18 to 20-hr fast to 100 min following feeding; (*c*) the decrease in FFA levels from the end of an 18 to 20-hr fast to 100 min following feeding. Values are means, or means ±S.E.

Changes in the levels of cortisol and FFA on days other than day 6 of each week did not change appreciably as the amount of feed offered increased, except for an expected decrease in FFA level as the feed per day increased from 300 to 900 g per day (Table 1). At the end of this experiment the sheep were fasted for 3 days. The results, shown in Figure 3, are characterized by highly variable changes in circulating cortisol levels but consistent rises in FFA as expected.

TABLE 1

EFFECT OF LEVEL OF FEED INTAKE ON SERUM LEVELS OF FREE FATTY ACIDS AND CORTISOL

The six sheep remained on each level of intake for 7 days

Feed offered per day (g)	Feed consumed per day (g)*	Serum cortisol (ng/ml)			Serum free fatty acid (μ-equiv./ml)		
		Day 3	Day 5	Day 7	Day 3	Day 5	Day 7
300	282	7·85 ±2·13	6·20 ±1·79	6·50 ±1·28	0·52 ±0·06	0·42 ±0·05	0·46 ±0·04
600	600	5·85 ±1·70	5·10 ±0·92	6·10 ±0·63	0·26 ±0·03	0·23 ±0·03	0·25 ±0·04
900	879	6·55 ±0·58	6·75 ±1·73	7·30 ±0·88	0·14 ±0·01	0·20 ±0·04	0·22 ±0·04
1200	1192	8·60 ±1·35	6·35 ±1·52	7·75 ±2·33	0·12 ±0·04	0·18 ±0·02	0·31 ±0·04
1500	1471	7·55 ±0·71	6·70 ±1·48	9·30 ±3·47	0·13 ±0·02	0·25 ±0·04	0·21 ±0·04
1800	1645	6·80 ±0·54	7·00 ±1·48	10·05 ±4·31	0·10 ±0·02	0·14 ±0·01	0·27 ±0·03
2100	1891	6·55 ±0·87	9·05 ±1·68	18·65 ±3·89	0·12 ±0·01	0·13 ±0·01	0·52 ±0·07

* Not including day 6.

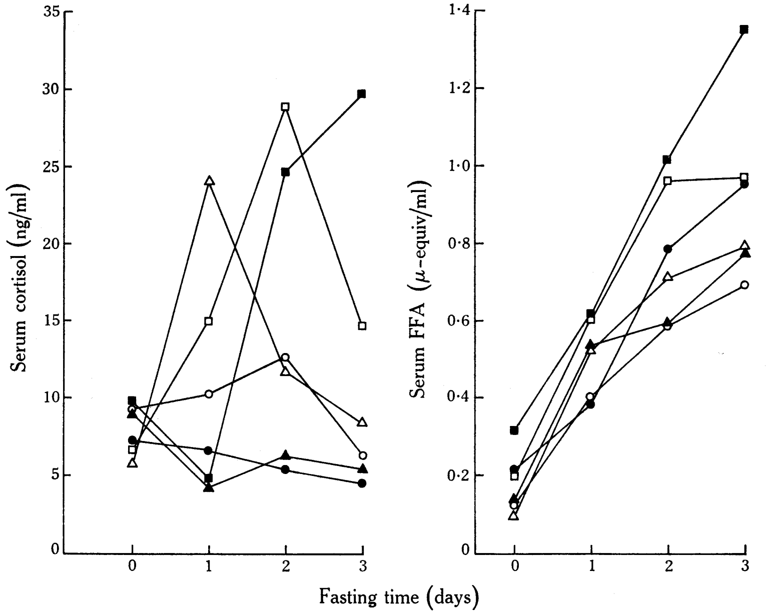

Fig. 3.—Changes in levels of serum cortisol and FFA in six sheep during a 3-day fast.

(*b*) *Weighing, Shearing, and Slaughter Stresses*

Because of the unsatisfactory nature of short periods of fast as a routine stress, other treatments were tested.

The first of these comprised a fast for 40–44 hr followed by preparation of the animals' feed, which was placed in front of them but out of their reach. Then they were weighed in a strange environment and bled as they were taken out of the scale. Changes in individual cortisol and FFA levels during this procedure are shown in Figure 4. There was a tendency for the highest cortisol levels to occur after the weighing, but there was considerable variation between animals. FFA levels, on the other hand, tended to be highest immediately prior to weighing.

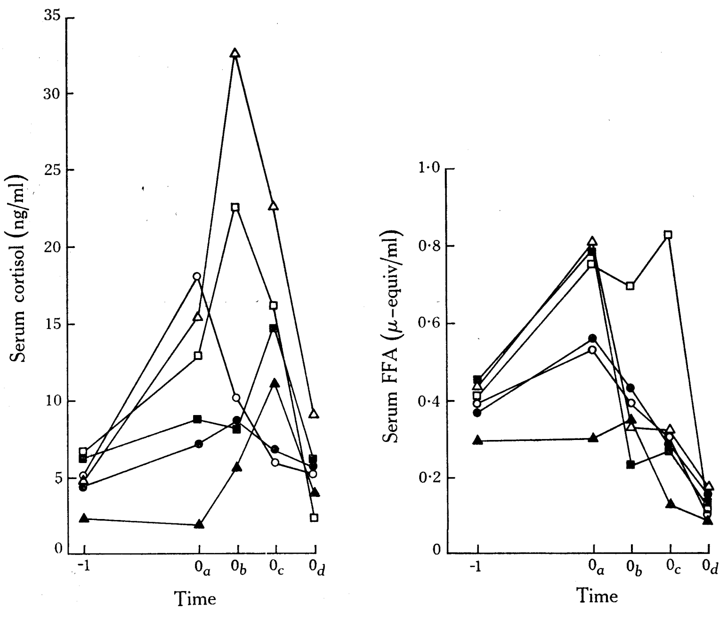

Fig. 4.—Changes in serum cortisol and FFA in six sheep at the following times: -1, levels following a routine 18 to 20-hr fast; 0_a, levels at the same time 24 hr later (42 to 44-hr fast); 0_b, levels following feed preparation and weighing of the sheep; 0_c, levels 5 min after feeding; 0_d, levels 100 min after feeding.

Concentrations of circulating cortisol and FFA immediately before and immediately after shearing are given in Figure 5. Over this period of approximately 10 min there was a consistent rise in cortisol levels but inconsistent changes in FFA levels. This is in contrast to the situation shown during the 3-day fast.

In order to find how the elevations in cortisol levels produced by these stresses compared with the cortisol levels normally encountered at slaughter under commercial conditions, blood samples were collected from 24 lambs as they were slaughtered at Homebush State Abattoir, Sydney. The distribution of cortisol concentrations is shown in Figure 6. The only values in the experimental sheep which approached these were those following shearing (Fig. 5), transportation (Fig. 9), or restraint (Table 2).

(c) *Reserpine Administration*

The first experiment involving reserpine administration was conducted immediately after an experiment in which a fast of 18–20 hr had been shown to elevate circulating cortisol levels. Reserpine was administered daily at 1, 4, 8, and 16 μg/kg

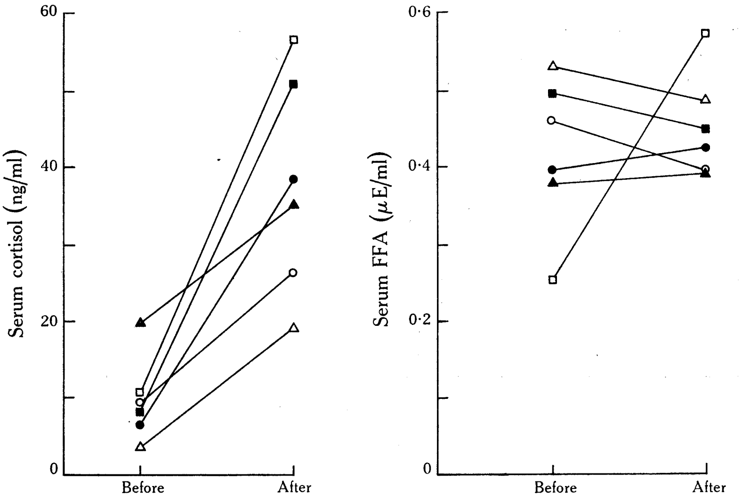

Fig. 5.—Effect of shearing on the levels of circulating cortisol and FFA in six sheep.

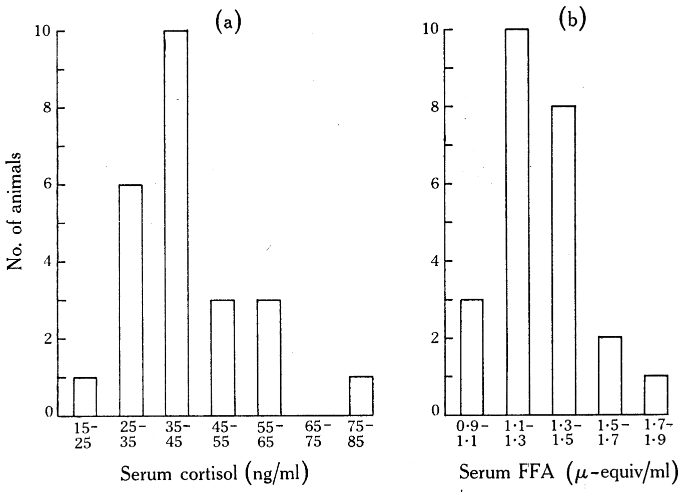

Fig. 6.—Distribution of serum cortisol and FFA levels in 24 lambs slaughtered at a commercial abattoir.

for 2 days, with cortisol levels being measured in pre-feeding serum samples daily from 1 day before administration to 2 days afterwards. There was a general decline in circulating cortisol levels as the level of reserpine administered increased but this was probably due to the animals becoming accustomed to the 18-hr fast, as levels did not rise during the 2 weeks following reserpine administration. However, at the

TABLE 2

EFFECT OF ADMINISTRATION OF RESERPINE FOR 3 DAYS ON CIRCULATING CORTISOL LEVELS AND ON THE RESPONSE OF CIRCULATING CORTISOL TO A RESTRAINING STRESS

The sheep were restrained for a 15–20-min period by tieing their feet together. Sample 1, at 9 a.m., 5 hr before restraint; sample 2, immediately before restraint; sample 3, immediately after restraint; sample 4, 30 min after sample 3; sample 5, 30 min after sample 4. Values are means ±S.E.

Daily reserpine dose (μg/kg)	Sample No.				
	1	2	3	4	5
Control	8·10	5·95	46·10	36·50	24·80
	±0·89	±0·81	±4·46	±10·70	±10·80
3	5·80	5·55	42·10	24·50	10·80
	±0·97	±1·01	±3·40	±3·59	±1·76
7	9·18	7·56	37·86	22·26	16·26
	±1·79	±1·56	±3·95	±5·56	±2·89

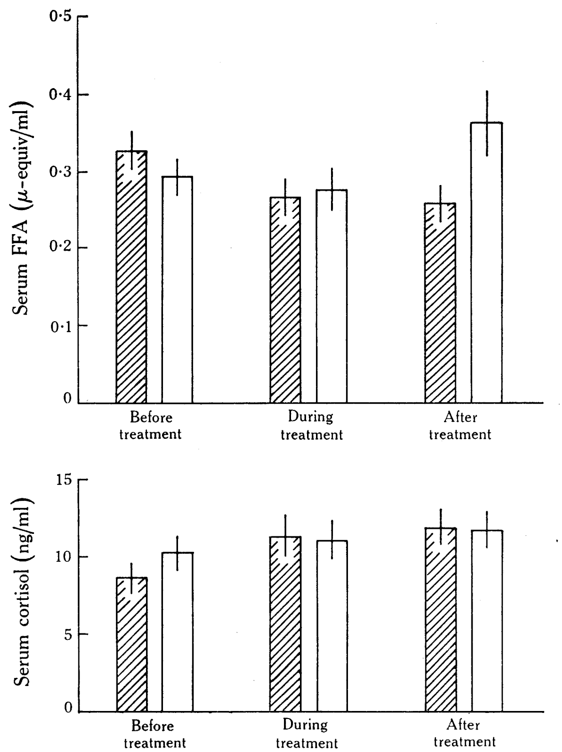

Fig. 7.—Levels of pre-feeding cortisol and FFA in sheep for 3 days each before, during, and after treatment with 5 μg/kg reserpine daily for 3 days. Values are means ± S.E. Hatched, treated sheep; open, controls.

highest level (16 μg/kg daily for 2 days) cortisol levels tended to be elevated during the treatment period and some animals became very drowsy.

In a second experiment, reserpine was administered at 5 μg/kg daily for 3 days and cortisol levels were monitored from 2 days prior to treatment to 4 days after it. A crossover design was used with the three control sheep in the first period being treated during the second period. Changes in serum cortisol and FFA levels are shown in Figure 7. There was no treatment effect on cortisol levels but the concentration of free fatty acids was significantly ($P<0\cdot05$) lower in the treated group during the post-treatment period. The patterns of change in circulating cortisol levels or FFA levels from before feeding to 100 and 200 min after feeding on day 5 were not affected by reserpine administration. These results suggest that reserpine at this level does not affect basal levels of cortisol in the sheep, but they do not indicate whether there was any effect on the response of cortisol levels to stress.

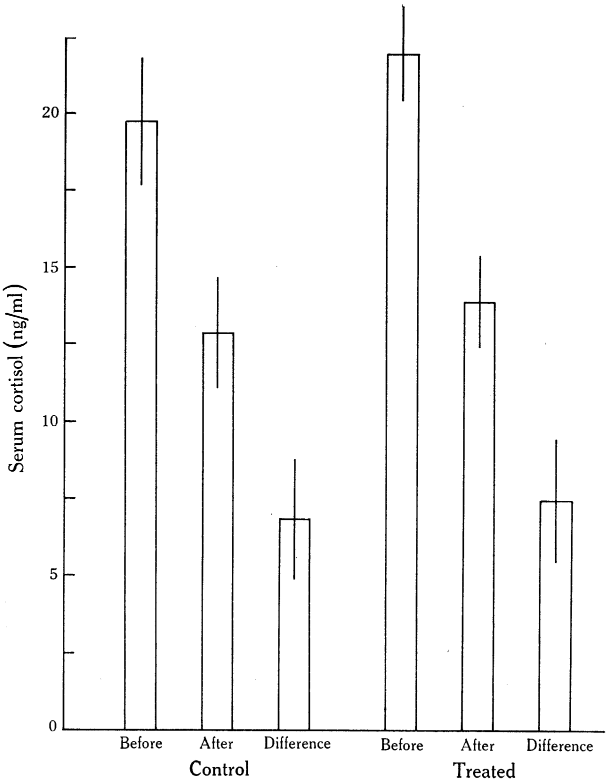

Fig. 8.—Changes in serum cortisol levels in lambs during their first few days in pens and the effect of reserpine administration on this change. "Before" values are for the mean of the first two samples taken. "After" values are for the mean of the third and fourth samples, both of which were taken after reserpine administration.

In order to test the stressed situation, reserpine was administered at the rate of 200 μg per animal daily (approximately 5 μg/kg daily) for 3 days to 12 Dorset Horn × Border Leicester × Merino lambs, of approximately 40 kg liveweight, which had been just weaned and transferred from pasture to individual pens. The lambs had not been bled previously. Two blood samples were taken on two days immediately prior to treatment and two were taken following treatment; one on the third day of reserpine administration and one on the following day. Twelve control lambs received the reserpine diluent. The results (Fig. 8) give no indication of any effect of reserpine on the difference between the cortisol levels in the first two blood samples and those in the second two.

A further test of the effect of reserpine at 5 μg/kg daily for 3 days on the response of cortisol to stress was conducted using the six wethers. A crossover design was used involving two approximately 7-min periods of transportation separated by an overnight stay in a strange pen. Figure 9 indicates that this treatment was effective in

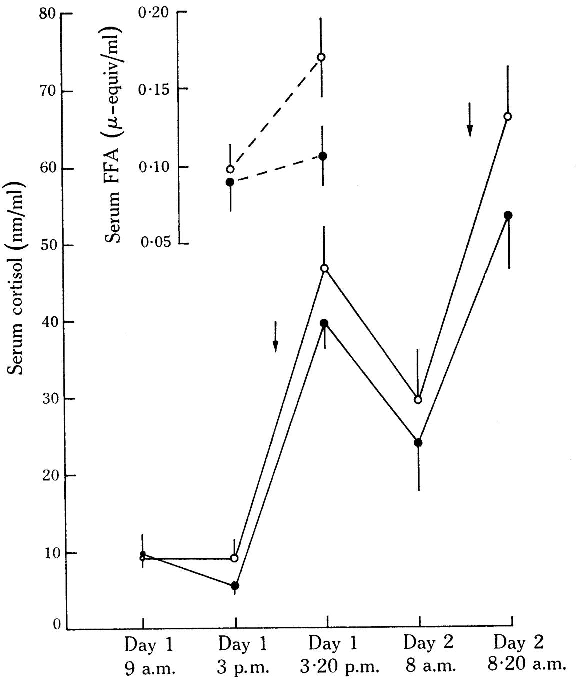

Fig. 9.—Changes in serum cortisol and FFA levels of sheep subjected to two 7-min periods of transportation and a stay in a strange environment. ○ Treated. ● Control. Vertical arrows indicate times when transportation commenced.

elevating cortisol levels but that reserpine administration did not suppress the response. The reserpine treatment did not affect the basal FFA levels but following transportation these levels were significantly higher in the treated group ($P < 0 \cdot 05$).

At this stage, an experiment was commenced which involved the same transportation stress, a three-way crossover design and levels of reserpine administration of 0, 10, and 15 μg/kg daily for 3 days. However, this experiment was discontinued after the first run as some sheep again became very drowsy and cortisol levels following transportation were considerably elevated in some of the treated animals (maximum 132·0 ng/ml). Also, the cortisol levels in the control animals were lower than in the previous experiment, which suggested that the sheep were becoming accustomed to the transportation.

In the next experiment, reserpine was administered at 0, 3, and 7 μg/kg daily for 3 days and on the third day the animals were restrained by their legs being tied together for a 15-min period. The effect of this treatment is shown in Table 2. There was no statistically significant effect of reserpine on basal cortisol levels, on the response of these levels to stress, or on the rate of decline of circulating cortisol levels after removal of the stress. However, there was a tendency for the cortisol peak in sample 3 to be lower in the groups receiving reserpine.

IV. Discussion

In order to test for an effect of a treatment on the response of circulating cortisol levels to stress, a representative stress which consistently elevates cortisol levels is required. Attempts to classify stresses have usually distinguished between those which cause ACTH release via neural pathways, such as sounds and strange environments (psychological stresses) (Brown *et al.* 1971), and those which act through non-neural pathways, such as circulating levels of histamine and serotonin (systemic stresses) (Ganong 1963; Mangili *et al.* 1966). In considering stresses which affect muscle, Lawrie (1966) placed emphasis on the psychological component and noted the wide variability in this component between animals. The stress of slaughter and pre-slaughter handling seems likely to be predominantly psychological in nature, so that in studying this period psychological stresses should be used.

(*a*) *Effects of Fasting on Cortisol Levels*

The elevations in cortisol levels which resulted initially from fasts of 18–20 hr is consistent with results involving other species including rabbits (Bouillé and Assenmacher 1970), rats (Slater 1962; Chowers *et al.* 1969), and man (Alleyne and Young 1967). In sheep no elevations in circulating corticosteroids on fasting were apparent from the results of Reid and Mills (1962) or Bassett (1968). In light of the very short fasting periods involved and of the loss of response with repeated exposure, it seems likely that the effect shown in Figure 1(*a*) is predominantly psychological in nature. The absence of comparable responses in other studies with sheep may be because the sheep were not being fed *ad libitum* prior to the fasts.

The loss of a cortisol response to a fast of 18–20 hr after the sheep were routinely fasted for this period each day is similar to the diminished cortisol response to repeated stresses that has been demonstrated in rats (Ada and Grota 1969). Also, within a period of fasting circulating cortisol levels may decrease after an initial rise (Bellamy *et al.* 1968; Bouillé and Assenmacher 1970).

Ganong (1963) reviews other instances of reduced response to repeated stresses, but in most cases the stresses were applied more frequently than once a day. The demonstration that this decreased response to fasting is to some extent regainable as the fasting regime becomes increasingly different from the usual feeding regime (Fig. 2) is a further indication that the animals had become accustomed to the fasting treatment.

(*b*) *Reserpine Administration and the Response to Stress*

In a review of this topic, Munson (1963) concluded that the response of circulating cortisol levels to stress was inhibited to some extent by reserpine. For example, Mahfouz and Ezz (1958) indicated that reserpine at a dose of 8 μg/kg inhibited the response of the rat to acute stress as measured by the degree of adrenal ascorbic acid depletion, while Mason and Brady (1956) demonstrated that corticosteroid elevations in rats in response to an anxiety stimulus were reduced by reserpine administration. It appears that this inhibition is greater when the reserpine is administered in multiple doses, a phenomenon which Munson (1963) attributed to depletion of pituitary ACTH supplies. Giuliani *et al.* (1966) pointed out that reserpine tended to increase basal levels of circulating ACTH levels in the rat, but that with reserpine treatment the response of ACTH to stress was diminished. They suggested that the former effect resulted from inhibition by reserpine of an inhibitory hypothalamic pathway, while the latter was mediated through an activation of the corticosteroid feedback mechanism. An alternative explanation advanced by Bhattacharya and Marks (1969) was that reserpine inhibited ACTH secretion at low doses but stimulated it at higher doses.

In the experiments reported herein, daily levels of 3–7 μg/kg for 3 days, or a total of 9–21 μg/kg, were chosen on the basis of the results of experiments which indicated that at a total dose of 30 μg/kg or more some sheep became drowsy and there was a tendency for cortisol levels to be elevated. The slight but significant ($P < 0 \cdot 05$) decrease in the level of FFA in the post-treatment period of one experiment (Fig. 7) suggests that reserpine did have a physiological effect, since in some studies on man (Davidson *et al.* 1971) it has been reported to lower FFA levels. This effect is also consistent with the general reduction in sympathetic tone brought about by reserpine (Adams *et al.* 1969). The results in Figure 9 do not fit into this pattern but in that experiment it was the changes in FFA level rather than basal levels which were affected by reserpine. In conclusion, it would appear that reserpine administered to sheep at the level of 9, 15, or 21 μg/kg in three equal-sized doses over 3 days does not affect or has very little effect on either the basal circulating cortisol levels or the degree to which these levels respond to stress.

(*c*) *Cortisol Levels and other Stresses*

The procedure involving a fast of $1\frac{1}{2}$ days, combined with tempting the animals with food and weighing them (Fig. 4), produced some elevation in cortisol levels and would probably be of some use in testing for the effect of a treatment on the response of cortisol levels to stress. However, animals may become accustomed to this procedure also if it is used frequently.

The proportional changes in circulating cortisol levels of sheep from basal levels to levels following shearing and at slaughter are similar to those reported by Kilgour and de Langen (1970). The fact that the absolute levels they reported were higher may be due to differences between assay techniques (Bowman and De Luna 1969; Beitins *et al.* 1970).

The levels of cortisol in the wethers following the second period of transportation (Fig. 9) are higher than the mean levels in lambs at slaughter (Fig. 6). This may be partially due to a phenomenon similar to that discussed by Levine and Mullins (1966), whereby rats which had been frequently handled showed greater elevations in circulating cortisol levels in response to stresses than those which had not been handled. Reid and Mills (1962), however, noted that the degree of elevation of cortisol levels during road transport was usually less in "trained" sheep housed indoors than in grazing sheep.

V. Acknowledgments

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