

Studies on the Specificity of Adrenocortical
Function in Ruminant Nutrition

1973

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Contents

Introduction	1
Chapter I. Studies on establishment of analytical method for glucocorticoids (11-OHCS)	4
1. Materials and Methods	
2. Results and Discussion	
(1) A quantitative method of plasma 11-OHCS level	
(2) A quantitative method of adrenal 11-OHCS level	
(3) Separation of cortisol and corticosterone	
(4) Precision of the methods obtained in this study	
Chapter II. Adrenal weights in various animals	24
1. Materials and Methods	
2. Results and Discussion	
Chapter III. 11-OHCS levels in both blood plasma and adrenal tissue of various animals	27
1. Materials and Methods	
2. Results and Discussion	
Chapter IV. Effect of season, age and sex on plasma 11-OHCS level	32
1. Materials and Methods	
2. Results and Discussion	
Chapter V. Effect of physical and psychological stresses on plasma 11-OHCS level	37
1. Materials and Methods	
2. Results	
3. Discussion	
Chapter VI. Effect of fasting on plasma 11-OHCS level	42
1. Materials and Methods	
2. Results	

3 Discussion

Chapter VII. Role of glucocorticoids on glucose metabolism in fasting animals	49
1. Materials and Methods	
2. Results	
3. Discussion	
Chapter VIII. Effect of ACTH on plasma 11-OHCS level and F/B ratio	59
1. Materials and Methods	
2. Results	
3. Discussion	
Chapter IX. Effect of ACTH, cortisol and corticosterone injection on blood eosinophils count.....	66
1. Materials and Methods	
2. Results and Discussion	
Chapter X. Effect of cortisol and corticosterone on blood glucose, protein and lipid level.....	74
1. Materials and Methods	
2. Results and Discussion	
Chapter XI. Effect of ACTH on growth and food intake.....	80
1. Materials and Methods	
2. Results	
3. Discussion	
Summary and Conclusion	88
References	95

Introduction

Thomas ADDISON (1855) called attention to a human syndrome (now called Addison's disease) associated with deterioration of the adrenal cortex. The disease appears to be the first clinical defect of an endocrine gland to be accurately described¹⁵⁴).

Over 30 different steroids have been isolated from the adrenal tissue up to date⁴⁸). However, the only 7 steroids are biologically active⁶⁹), and that the only following; cortisol, corticosterone and aldosterone, are normally secreted in physiologically significant amounts⁴²).

These steroids are classified roughly into two groups; glucocorticoids and mineralocorticoids. The former are the steroids in which effects on glucose and protein metabolism predominate, while the latter those in which effects on sodium and potassium excretion predominate⁴²).

The physiological actions of glucocorticoids are broadly divided into two categories as follows; 1) to protect an animal body from a stress, i.e., when an animal is exposed to a stressor, the body responds towards adaptation¹³²). Increase of plasma 11-OHCS is one of the signs of such responses. ; 2) to control some intermediary metabolism, especially gluconeogenesis^{30,41,51,53,99,118,157}).

The adrenal cortex is controlled by hypothalamic-pituitary system. The ACTH secreted from pituitary stimulates the adrenocortical function and increases the secretion of glucocorticoids. As the result, when the plasma glucocorticoids are elevated, the anterior pituitary diminishes the output of ACTH^{68,156}).

As well known, the specific digestion, absorption and metabolism, especially on carbohydrate, are run in ruminants; glucose is absorbed from gut as a major end product of the ingested carbohydrate in most of animals, while volatile fatty acids produced by micro-organisms in reticulo-rumen⁵⁰), are absorbed as the products in ruminants^{3,52,74}).

Glucose is essential for nervous conductivity in mammals^{55,67,158}). The respiratory quotient (R.Q.) in brain is 0.97 to 0.99, indicating that the main energy source is glucose⁴⁰) as supported by MCCOLYMONT *et al.*⁸⁷). Moreover, about 70 % of the circulated glucose from the liver is utilized by the brain in humans⁹⁴), and a similar value could be true for ruminants⁷⁵). It was stated that the transfer rate of glucose expressed as a function

of metabolic body size ($W^{3/4}$) in ruminants was comparable to that in nonruminants¹⁰⁾.

These two facts in regard to absorption and utilization of glucose indicate that a great amount of glucose required as an energy source must be synthesized from non-carbohydrate precursors in ruminants.

Since there is no storage of carbohydrate in brain⁶⁴⁾, the utilized glucose is largely supplied from the circulatory blood glucose. It depends on the level of blood glucose whether the supply is sufficient or not⁸⁰⁾. MCCLYMONT *et al.*⁸⁷⁾ were unable to demonstrate any utilization of acetate by arteriovenous difference studies on the brain of sheep, though the acetate was comparatively rich in the circulating blood of ruminants¹¹³⁾. It is also suggested from these points that gluconeogenesis may be of particular importance in ruminants with the low blood glucose level even normally. Also, this may be supported by the following fact. The glucose tolerance in ruminants is generally lower than that in non-ruminant animals. MCCANDLESS and DYE⁸⁵⁾ postulated that a high level of gluconeogenesis in the ruminant was the primary factor which determine the low tolerance.

Glucocorticoids are directly related to regulate the process for gluconeogenesis^{30,41,51,53,99,118,157)}, though many hormones; insulin, glucagon, epinephrine, growth hormone, etc., have important roles in the regulation of carbohydrate metabolism⁴¹⁾.

Therefore, the gluconeogenetic role of adrenal cortex may be more important in ruminants than in non-ruminant animals. As an evidence, it has been indicated that metabolic disorders in pregnancy toxemia and ketosis were characteristic of ruminants, and their causes might be directly or indirectly mediated by the function of adrenal cortex regulating carbohydrate metabolism^{114,115,133)}.

As compared with the cases of humans and the laboratory animals, there was little basic information available concerning the adrenocortical function in ruminants. Then, the author intended to investigate basically characteristics of adrenal cortex in ruminants from the comparative viewpoint.

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Chapter I. Studies on establishment of analytical method for glucocorticoids (11-OHCS)

It has been known that more than thirty kinds of steroids exist in adrenal cortex, though all these hormones are named generically as adrenocorticoids⁴⁸⁾. Cortisol and corticosterone, so called 11-hydroxy-corticosteroids (11-OHCS), have the highest activity to carbohydrate metabolism in adrenocorticoids^{42,52,69)}. Content of these hormones in either blood or adrenal glands is very small and they are labile to high temperature. Therefore, 11-OHCS levels in the living body were not determined easily, but many kinds of new methods have been improved recently.⁷⁹⁾

In addition the relative amounts of cortisol and corticosterone vary widely among species, though either cortisol or corticosterone (or a mixture of both) is uniformly present in blood of various species^{19,45)}. Moreover, it is also suggested that there may be a difference between action of cortisol and that of corticosterone⁴²⁾. From such reasons, it is necessary to consider not only the total level of 11-OHCS, but also the ratio of cortisol to corticosterone (F/B ratio) in plasma and adrenal glands. However, no routine method for separation of cortisol and corticosterone is available yet.

The first purpose in this study is to obtain an microquantitative method for 11-OHCS levels in both plasma and adrenal tissue of domestic animals. The second is to get a method for determination of the F/B ratio. The third is to confirm accuracy of the methods obtained.

1. Materials and Methods

a) Sample;

Blood samples were taken from the jugular vein of cattle and sheep, from the heart of rabbits and from the cervical artery of decapitated domestic fowls and rats. Heparin sodium was added as an anticoagulant. The heparinized blood was centrifuged for 20 minutes and the supernatant plasma was stored at -20°C . The adrenal glands removed from animals were immediately trimmed, weighed and stored at -20°C .

The blood plasma and adrenal glands of cattle were offered from the

public slaughter house of Kyoto city. Sheep, rabbits and rats had been fed at the experimental farm in Kyoto University.

b) Reagent;

An extra pure reagent of methylene chloride was distilled at 38.5°C prior to use. And the methylene chloride used for analysis was distilled and used again. Ethanolic-sulfuric acid (75 V/V %) was prepared by slowly adding one volume of ice-cold ethanol to three volumes of ice-cold concentrated sulfuric acid (Guaranteed Reagent), the mixture being cooled in ice-cold water. This mixture was freshly prepared at each experiment. Fluorescence was unstable unless the temperature of the solution was prevented from elevating at the preparation.

c) Standard hormone;

Cortisone, Deoxycorticosterone, Corticosterone (Merk) and Cortisol (Takeda) were used as standard hormones in this study.

d) Instrument and apparatus;

It is essential to keep all glasswares absolutely clean. Such treatments as rinsing with chromic acid overnight, the following rinsing with water and finally washing with distilled water, were found to be adequate.

Glass-stoppered tubes should be used for all extractions at any cost. If a plastic stoppered tube was used, it was impossible to measure the optical density of glucocorticoids at the wave length of 240 m μ , at which peak absorption in the hormones occurs, because some substances extracted from the plastic stopper by methanol had remarkable absorption at the wave length under 290 m μ (Fig. 1).

A Hitachi Spectrophotometer-124 type and a Shimadzu-Kotaki Microfluorometer-UM type were used.

e) Preparation of thin layer plate;

Thin layer plates were prepared following STHAL's standard method⁶⁰). The outline is as follows; a suspension was made from 30 g of Kiesel gel H (Merk) and 65 ml of distilled water by triturating in a mortar. This suspension was placed in an applicator (Yazawa Scientific

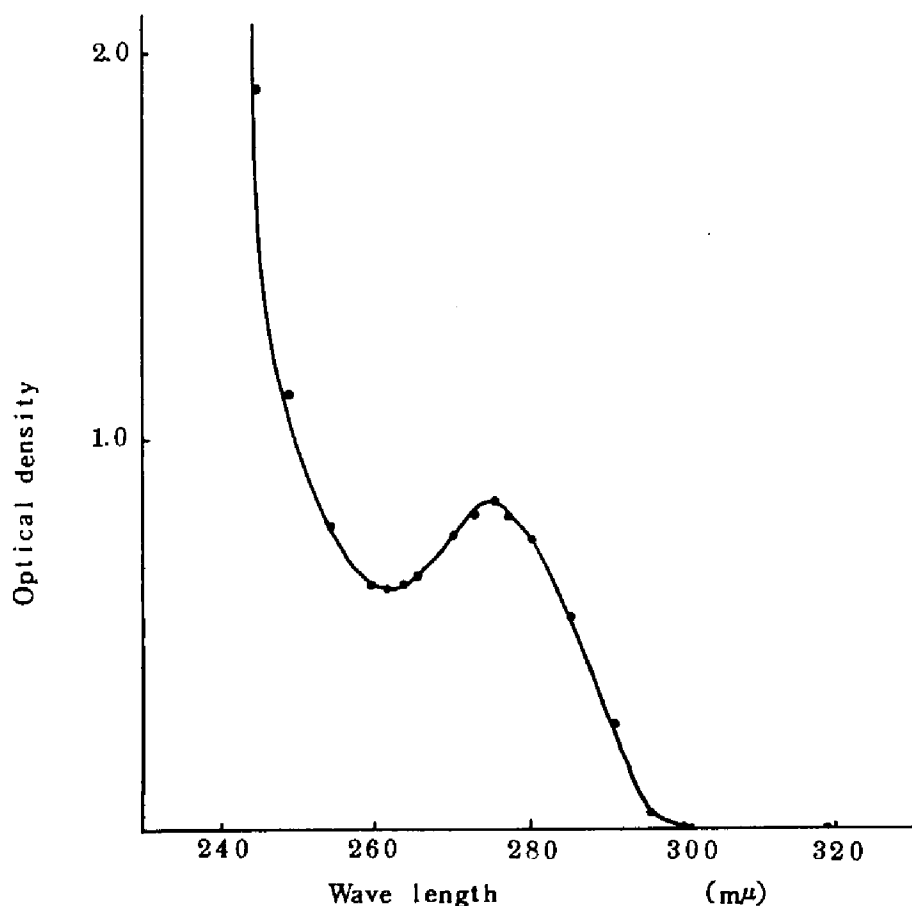


Fig. 1 Absorption curve of substances extracted from a plastic stopper by methanol

Instrument Co.) and applied onto 4 pieces of glass plate, 20 x 20 cm, to make a thin layer of 250 μ in thickness. They were allowed to stand about 15 minutes in the atmosphere and dried at 130°C for 1 hour to activate. The plate was preserved in a tightly closed vessel.

2. Results and Discussion

(1) A quantitative method of plasma 11-OHCS level

a) Extraction of 11-OHCS from plasma

An extractive solvent must be selected with which only 11-OHCS

are extracted as much as possible. Regarding these points, methylene chloride seemed to be the most superior solvent.

The extractive procedure was investigated, using methylene chloride as the solvent. The recovery with standard deviation of cortisol (0.8 μ g)

Table 1. Recoveries of cortisol and corticosterone added to plasma

Animal	Sample volume (ml)	Amount added (μ g)	Number	Recovery (%)	
				Cortisol	Corticosterone
Cattle	10	0.8	2	112.2	
		0.4	2		98.2
Cattle	4	0.8	8	97.4 $\pm$ 9.0 ^{a)}	
		0.4	12		101.2 $\pm$ 10.4 ^{a)}
Cattle	4	0.4	2	98.5	
		0.2	2		100.6
Rabbit	4	0.8	2	97.7	
		0.4	2		108.6

a): Mean $\pm$ standard deviation of the mean

or corticosterone (0.4 μ g) added to 4 ml of plasma of cattle was 97.4 $\pm$ 9.0 % or 101.2 $\pm$ 10.4 %, respectively (Table 1). These recoveries were quantitatively sufficient and their standard variations were lower than those by Braunsberg¹⁵⁾; the recovery of cortisol was 98.3 $\pm$ 1.74 %. Finding that the large variation of recoveries especially depended on the technique of shaking for extraction, the variation was improved to be smaller.

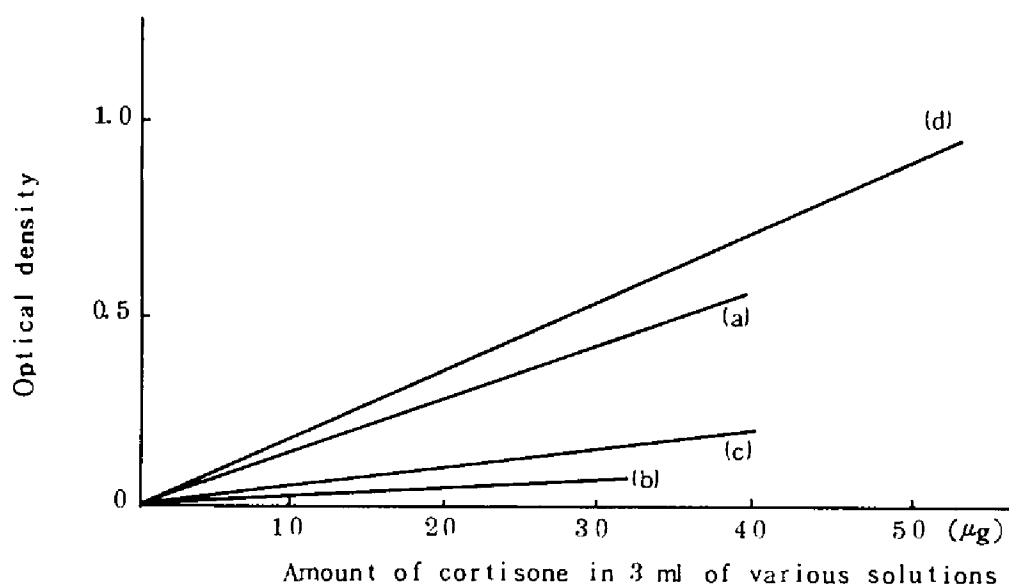
The results of Table 1 also indicated that 11-OHCS in plasma could be extracted to the similar extent by the present method and technique, regardless of the various volumes of plasma, the various plasma 11-OHCS levels and plasma samples of the different animal species.

b) Measurement of 11-OHCS

The various methods for measuring adrenocorticoids have been considerably

improved for these ten years. These methods can be classified into the following three classes; colorimetric procedure^{23,27,78,110,169,170}, fluorometric procedure^{47,93,137}) and radioisotopic procedure^{7,36}).

A comparison of the sensitivity among some colorimetric methods was carried out in the first instance. As shown in Fig. 2, sulfuric acid chromogen method¹⁶⁹) and ultraviolet (U.V.) absorption method¹⁷⁰) were more spectrophotometrically sensitive than the others.



- (a): U. V. Absorption Method
- (b): Diphenylamine Reaction Method
- (c): Porter-Silber Reaction Method
- (d): Sulfuric Acid Chromogen Method

Fig. 2. Absorbance of cortisone by various spectrophotometric methods

Although it was tried to measure the final extract from 10 ml of plasma of cattle, the hormones were too little to be detected even by the two colorimetric methods. The absorption curve of the extract from about 100 ml of plasma did not have peak absorption specific for glucocorticoids. The extract from larger amount of plasma was purified by

thin layer chromatography and cortisol and corticosterone fractions were separated. The absorption curves of the two fractions by sulfuric acid

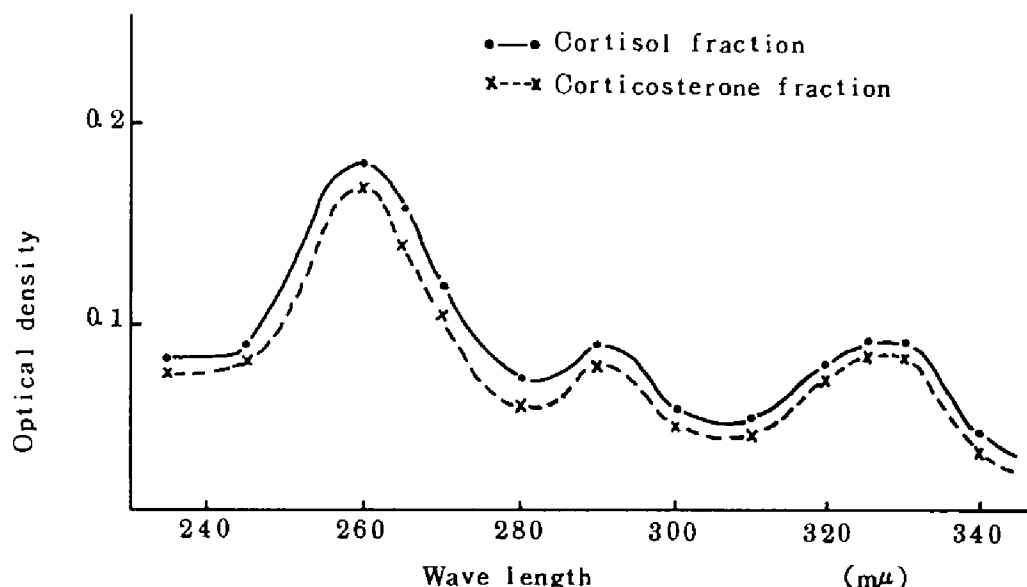


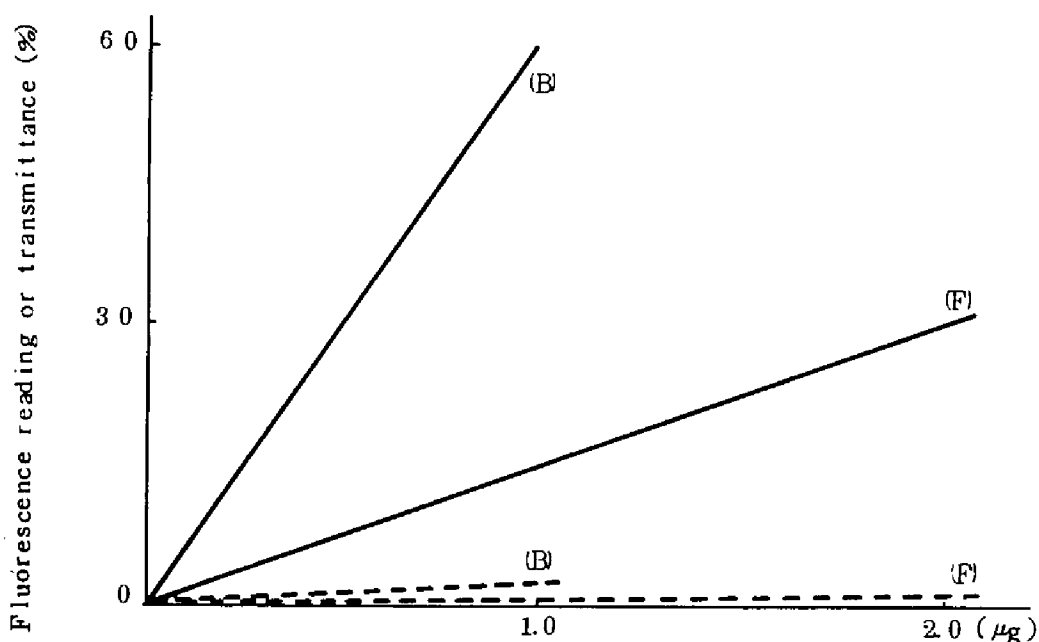
Fig. 3. Absorption curve of the substances extracted from plasma and purified by thin layer chromatography by sulfuric acid chromogen method

chromogen method were as shown in Fig. 8, indicating that they were identical to the curves of cortisol and corticosterone¹⁶⁹⁾.

The results suggested that these colorimetric methods were practically lacking in sensitivity and specificity for glucocorticoids. Therefore, such methods were not probably adequate to routine analysis because they would need a great amount of plasma and much time.

Secondly, the sensitivity of U.V. absorption method¹⁷⁰⁾ was compared with that of fluorometric method advanced recently¹⁴⁷⁾. The results indicated that the latter was strikingly more photometrically sensitive than the former, as shown in Fig. 4.

Fluorescence of the major adrenocorticoids was measured as shown in Table 2. Fluorescence readings of cortisol and corticosterone were



Amount of (B) or (F) in 5 ml of solutions

(B) : Corticosterone; (F) : Cortisol

— : Fluorometric Method

--- : Spectrophotometric Method

Fig. 4. Fluorescence reading and transmittance of corticosterone and cortisol by fluorometric and spectrophotometric methods

Table 2. Relative fluorescence of adrenocorticoids

Item	F.R./ μg^a)	Exponent
Corticosterone	8.20	1.00
Cortisol	4.78	0.58
Deoxycorticosterone	0.35	0.4
Cortisone	0.09	0.1

a): This was converted into the fluorescence reading per 1 μg of steroid

extremely high, while the others were only faint.

It was suggested from these results that the fluorometric procedure was the most adequate to determine the minute level of 11-OHCS in either plasma or adrenal glands.

Conditions for fluorometric measurement of 11-OHCS with Shimadzu-Kotaki micro-fluorometer-UM type were investigated.

When the ethanolic-sulfuric acid solution contained either cortisol or corticosterone was maintained at high temperature, the fluorescence reading rapidly increased and reached a maximum at the early time, and decreased very fast. When it was maintained at low temperature in the reverse, the reading slowly reached the maximum. It was suggested from these results that about 20 °C was the most adequate as temperature for the standing.

As shown in Fig. 5, fluorescence readings of cortisol and corticosterone

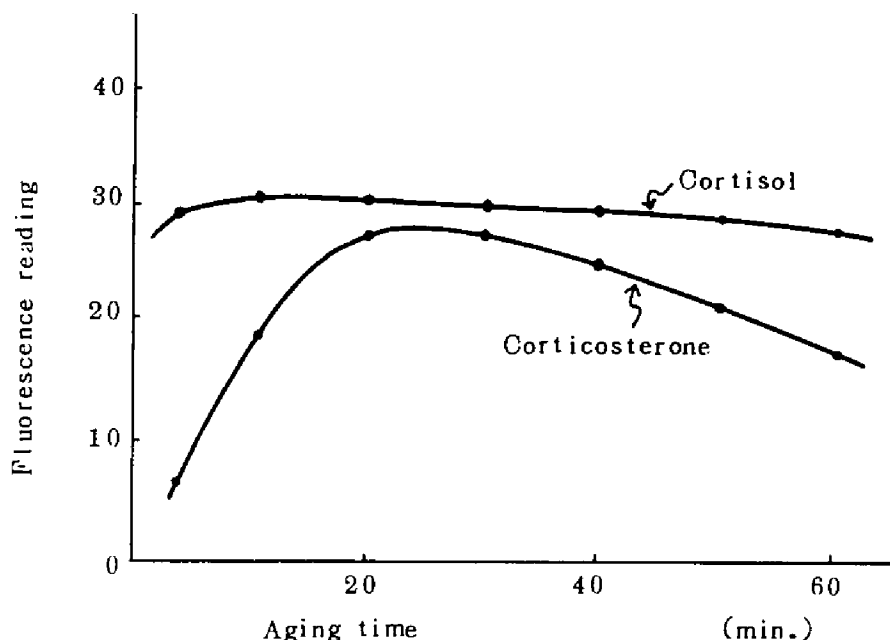
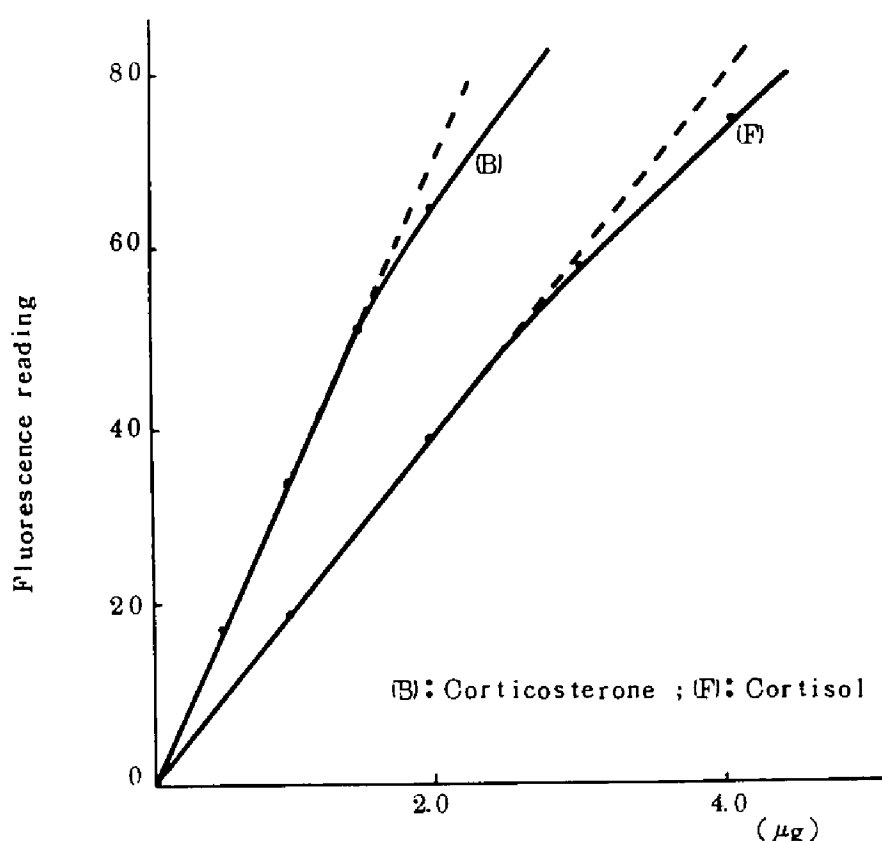


Fig. 5. Effects of aging time on the fluorescence of corticosterone and cortisol

varied as the time proceeded at 20 °C. Fluorescence reading of cortisol reached a plateau at about 5 minutes after addition of ethanolic-sulfuric acid and remained high level for a long time, while that of corticosterone was elevated slowly and reached a maximum at 20 minutes, and began to decrease at 40 minutes after the addition. Based on these results, fluorescence of either cortisol or corticosterone was measured at 30 minutes after the addition.

Under these conditions, relationships between amounts of either cortisol or corticosterone and the fluorescence readings were as shown in Fig. 6. The relationships fitted the law of Lambert-Beer, if the



Amount of (B) or (F) in 5 ml of ethanolic-sulfuric acid solution

Fig. 6. Standard curves of corticosterone and cortisol

amounts were less than $2.0 \mu\text{g}$ per 5 ml of ethanolic-sulfuric acid in cortisol and less than $1.0 \mu\text{g}$ in corticosterone. But self-absorption effect of the fluorescence was observed in the case of above each level. This result was in approximate agreement with the report by SWEAT¹⁴⁷⁾ that cortisol more than $6.5 \mu\text{g}$ resulted in self-absorption effect. A relationship between amounts of each hormone and the fluorescence readings was linear and useful for the measurement in the case of smaller amount than each limit; $2.0 \mu\text{g}$ of cortisol or $1.0 \mu\text{g}$ of corticosterone per 5 ml of ethanolic-sulfuric acid. An amount of either cortisol or corticosterone used for standard was $0.8 \mu\text{g}$ or $0.4 \mu\text{g}$, respectively.

c) A quantitative method of plasma 11-OHCS level

The outline of the procedure is shown in Fig. 7. Plasma samples

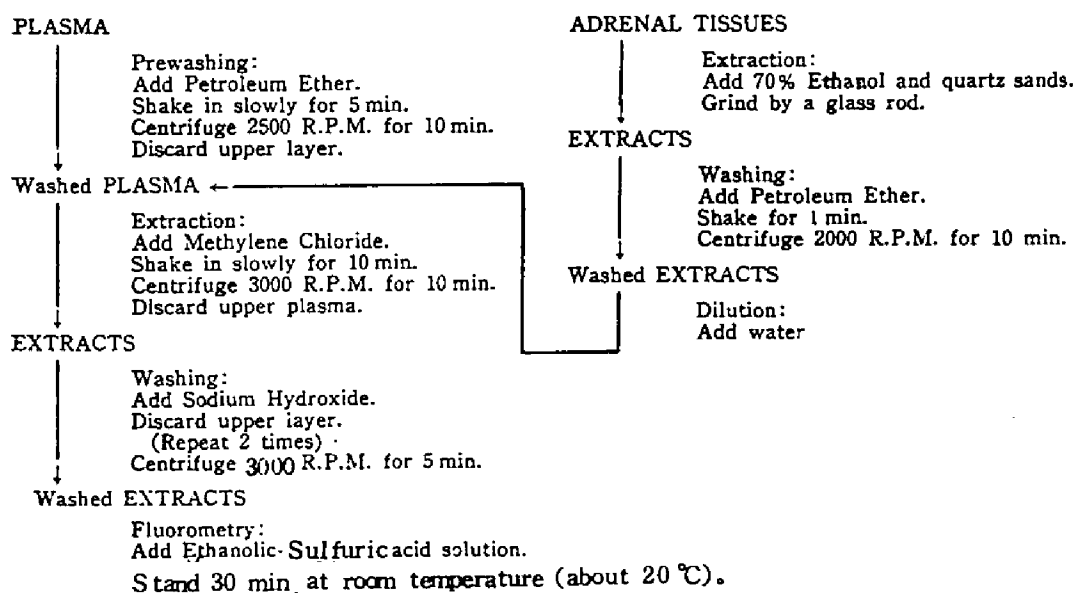


Fig. 7 The determination of 11-OHCS in plasma and adrenal tissues

were poured into glass-stoppered centrifugal tubes and were shaken for 5 minutes with the three volumes of petroleum ether. A particular attention was needed at the shaking because plasma layer could not be easily separated from petroleum ether, if the shaking was too vigorous. After centrifugation (2500 r.p.m.) for 10 minutes, the petroleum ether layer was discarded. The washed plasma (a ml) was transfused into the other new centrifugal tube or a separatory funnel and shaken for 10 minutes with the five volumes of methylene chloride (b ml). If the shaking was too vigorous, methylene chloride and plasma were inseparable. If too weak, *vice versa*, either cortisol or corticosterone added to plasma were not recovered completely. It was required that a constant shaking intensity was always kept. After the shaking, it was centrifuged for 10 minutes again. When a volume of the plasma was less than 4 ml, methylene chloride extract was easily separated from the plasma layer. After the upper plasma layer was discarded, the lower extract layer was shaken vigorously for 30 seconds with the 1/15 volume of 0.1 N sodium hydroxide, and allowed to stand until the separation into two layers appeared clearly. After removal of the upper sodium hydroxide layer, the lower extract was shaken with the sodium hydroxide solution in the same way as the previous case, and centrifuged with 3000 r.p.m. for 5 minutes.

After the removal of the upper layer, methylene chloride extract (c ml) was transfused into the other new centrifugal tube. This extract cooled in ice-cold water was shaken for 20 seconds with 5 or 4 ml of ice-cold ethanolic-sulfuric acid solution and allowed to stand in ice-cold water for a few minutes until the mixture was definitely divided into two layers. The upper methylene chloride layer was removed carefully. The lower layer was allowed to stand 30 minutes at the room temperature (about 20°C) and measured by the fluorometer. At that time, 1F-470 mμ and a combination of UV-O₂, and FL-B₂ or FL-530 mμ were used as the primary filter and the secondary filters, respectively.

Plasma 11-OHCS level (M μg/100 ml) was calculated by means of the following formula;

$$M = P \times \frac{X - X_o}{X_s - X_o} \times \frac{b}{c} \times \frac{100}{a}$$

- X : Fluorescence reading of a sample
 X_0 : Blank - fluorescence reading of only the residue of methylene chloride
 X_s : Standard - fluorescence reading of a certain hormone (P μ g) and the residue of methylene chloride

(2) A quantitative method of adrenal 11-OHCS level

To extract 11-OHCS from the ground adrenal tissue, HAINES^{'49)}, MACCHI^{'s 77)} and GUILLEMIN^{'s 46)} methods have been known up to date. HAINES^{'49)} method is complicated and takes much time. The latter two methods^{46,77)} are suitable to extraction of corticosterone like corticoids, but not to that of cortisol⁹⁸⁾.

A method for extraction of both cortisol and corticosterone was investigated in this experiment, based on the suggestion of NISHIKAZE⁹⁸⁾.

The outline of the method for determination of adrenal 11-OHCS level is shown in the right half of Fig. 7. Adrenal tissue (a mg) was placed into a centrifugal tube and ground by a glass rod with the help of quartz sands. Ten ml of 70 % ethanol per g of adrenal tissue was added and the tissue was ground thoroughly. It was vigorously shaken for one minute with the three volumes of petroleum ether and centrifuged at 2000 r.p.m. for 10 minutes. The upper petroleum ether layer was discarded. At this time the lower ethanolic layer should not be removed even a little. Content of ethanol in the extract was adjusted to 30 % with water. The final volume was b ml. After shaking gently, the extract was centrifuged at 2000 r.p.m. for 10 minutes again and the remaining petroleum ether and floating substances were discarded. This extract (c ml) was re-extracted with d ml of methylene chloride. And the methylene chloride re-extract (e ml) was measured fluorometrically as described in the measurement of plasma 11-OHCS.

Adrenal 11-OHCS level (N μ g/100 mg) was calculated by the following formula;

$$N = P \times \frac{X - X_0}{X_s - X_0} \times \frac{b}{c} \times \frac{d}{e} \times \frac{100}{a}$$

X : Fluorescence reading of a sample

X₀ : Blank — fluorescence reading of only the residue of methylene chloride

X_s : Standard — fluorescence reading of a certain hormone (P μ g) and the residue of methylene chloride

Recoveries of cortisol and corticosterone added to adrenal tissue were $85.3 \pm 11.1 \%$ and $108.0 \pm 12.0 \%$, respectively. Although the recovery of cortisol was somewhat low, the method was considered to be utilizable, so far as it was used for a comparison among animal species.

(3) Separation of cortisol and corticosterone

Since both cortisol and corticosterone in plasma and adrenal glands were very low levels and thermolabile, it was considered to be difficult to isolate each hormone from such samples in the past. Recently, thin layer chromatography (TLC) has been recognized as a valuable analytical tool. This seemed to be superior, regarding separability, sensitivity, developmental rapidity and simplicity of the apparatuses and the procedures than the other chromatography. Then, TLC was utilized in order to isolate cortisol and corticosterone independently from the extracted residue of plasma or adrenal glands.

a) Development

It was reported that the mixed solvents of two or more reagents were superior as the developing solvent for separation of adrenocorticoids^{76, 86, 112, 149, 160}). These solvents were compared with each other regarding stability and separability. As the results, chloroform-methanol solvent (90 : 10, V/V) was adopted.

As shown in Fig. 8, R_f values of cortisol and corticosterone with the solvent declined as the time proceeded and became constant in one hour after pouring the solvent; 0.46 ± 0.03 and 0.57 ± 0.03 , respectively, indicating that saturation time of the developing solvent was one hour.

b) Detection

For detection of adrenocorticoids on thin layer plate, sulfuric acid spraying method¹⁴⁹), antimon-trichloride method¹⁴³), fluorescein-sodium

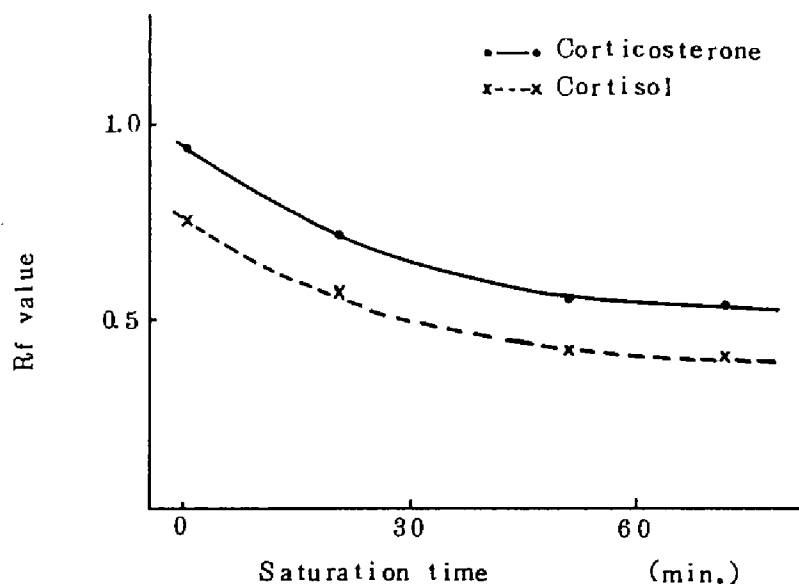


Fig. 8 Effect of the saturation time on the Rf value of corticosterone and cortisol

method⁸⁶⁾, tetrazolium blue method¹⁶⁰⁾, iodine-vapour method⁸¹⁾, vanillin method⁸²⁾, etc., have been developed.

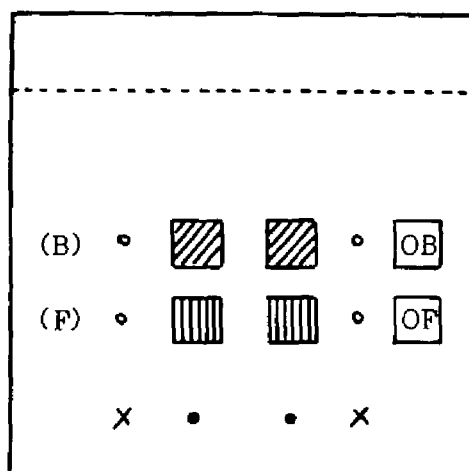
Detectable limits of deoxycorticosterone and cortisone by three methods^{81,82,86)} were shown in Table 3. It was reported that antimon-

Table 3. Minimal detectable amount of deoxycorticosterone and cortisone on TLC by three methods

Substance	Fluorescein Na	Vanillin	Iodine
Deoxycorticosterone	0.5	0.5	1.0
Cortisone	1.0	2.5	2.5

Unit: μ g; Diameter of spots : Ca. 5 mm

trichloride¹⁴³⁾ and sulfuric acid spraying method¹⁴⁹⁾ were more sensitive than these methods; even 0.1 μg of an adrenocorticoid could be detected by both the methods. The sensitivity of iodine-vapour method was not so high as shown in Table 3. However, the part of double bonds in the molecule of an adrenocorticoid binds loosely with iodine and shows a visible color, and this color disappears gradually. And the property of the hormone is not changed through this procedure⁸¹⁾. The fluorescence reading of 11-OHCS was not affected by the spraying of iodine-vapour. Therefore, the iodine-vapour method seemed to be superior for the quantitative determination of the hormone after the detection. Fig. 9 showed the portions of cortisol and corticosterone in a sample presumed from those of the detectable amount of standards by the spraying of iodine-vapour.



- : Extracts from sample
- × : Standards which contains cortisol (F) and corticosterone (B)

Fig. 9 Portions of cortisol and corticosterone presumed, based on the Rf values of standards

c) Elution

It had been difficult to isolate purely and quantitatively a substance from the 'silica gel'. The difficulty had prevented TLC from being utilized to a quantitative analysis. Some methods have been proposed for the elution^{81,91,96,141)}, but their methods had any defects. The modified MATTHEWS' method was used in this study. The apparatus was shown in Fig. 10.

If the elute was dried by the help of N_2 gas flow on water bath, the recovery was not high. The residue might be blown off by the gas flow, attaching to corpuscles of silica gel. This difficulty was overcome by the next procedures; 3 volumes of the ice-cold sulfuric acid were slowly added to 4 volumes of the ice-cold elute of methylene chloride-ethanol (3 : 1, V/V), and shaken vigorously for 20 seconds. Such mixed solution was allowed to stand in ice-cold water until two layers appeared clearly. The upper methylene chloride layer was discarded. The lower layer (75 % ethanolic-sulfuric acid contained the extracted hormones) was allowed to stand 30 minutes at about 20 °C, and was measured by means of the fluorometer.

Recoveries of cortisol and corticosterone added on TLC plate by the modified elution method are shown in Table 4. The recoveries without development and detection were 91.7 % and 90.8 % in cortisol and corticosterone, respectively. Recovery did not change even if more amounts of a hormone were applicated on thin layer plate. The recovery with development and detection was slightly lower than that without development and detection, but no difference between the recovery of cortisol and that of corticosterone was appreciated.

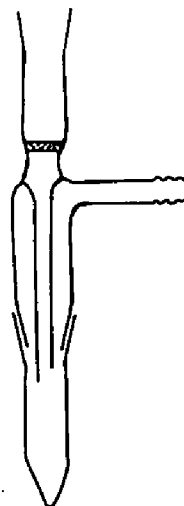


Fig. 10. Elution apparatus for the isolation of cortisol and corticosterone from the 'silica gel'

Table 4. Recoveries of cortisol and corticosterone obtained by elution from TLC plate of Kieselgel H

Item	Amount added (μg)	Recovery (%)	
		Pre-Development	Post-Development
Cortisol	2.0	90.8 (2) ^{a)}	
	0.8	91.7 (3)	81.0 $\pm$ 9.7 ^{b)} (5)
Corticosterone	1.0	89.5 (2)	
	0.4	90.8 (3)	81.5 $\pm$ 13.8 (5)

a): Each number in parentheses shows the number of replications

b): Mean $\pm$ standard deviation of the mean

It was suggested, therefore, that the modified elution method could be used in order to estimate F/B ratio in plasma and adrenal glands.

d) A method by means of TLC for separation of cortisol and corticosterone

The outline of the obtained method is as follows; a methylene chloride extract from either plasma or adrenal tissue was dried by flowing N_2 gas on water bath (40 – 50 °C). This residue was dissolved in a little amount of methylene chloride and spotted on thin layer plate at a position of 2.0 cm above the lower end of the plate using a glass capillary. The standard mixture of cortisol and corticosterone was also dissolved in methylene chloride and spotted on the same line of both sides of the sample. After the solvent was thoroughly evaporated, the thin layer plate was put quietly into a developing vessel which had been saturated with a vapour of the developing solvent – chloroform and methanol (90 : 10, V/V).

In order to facilitate saturation, a piece of filter paper soaked in the developing solvent was placed inside the vessel. The development was done by ascending solvent chromatography at room temperature and completed when the solvent moved 10 cm upwards. The plate was taken

out and dried.

Iodine-chloroform solution (0.5 %) was sprayed on the plate and the positions of cortisol and corticosterone were presumed from those of standards as shown in Fig. 9. The 'silica gel' of the positions drawn oblique or horizontal line was transferred on the apparatus (Fig. 10) and washed with a mixed solvent of methylene chloride and ethanol (3 : 1, V/V) until the elute reached 5 ml.

This elute was cooled in ice-cold water and 3.75 ml of sulfuric acid was added gently. It was vigorously shaken for 20 seconds and allowed to stand in ice-cold water until two solvent layers appeared clearly. After removal of the upper methylene chloride layer, fluorescence of the lower layer was measured under the same conditions as described at the determination of plasma 11-OHCS level.

E/B ratio was calculated according to the following formula;

$$E/B \text{ ratio} = \frac{X_f - X_{of}}{X_b - X_{ob}} \times \frac{\frac{q}{X_{sf}}}{\frac{p}{X_{sb}}} = \frac{qX_{sb}(X_f - X_{of})}{pX_{sf}(X_b - X_{ob})}$$

X_f or X_b : Fluorescence reading of cortisol or corticosterone fraction in a sample, respectively

X_{of} or X_{ob} : Blank - fluorescence reading of the position OF or OB in Fig. 9, respectively

X_{sf} or X_{sb} : Standard - fluorescence reading of cortisol ($q \mu g$) or corticosterone ($p \mu g$), respectively

(4) Precision of the methods obtained in this study

The determination of 11-OHCS level was repeated twice on the same plasma sample. The difference between the two values was 4.3 ± 3.2 % of the mean. A value of plasma 11-OHCS level obtained by the method seemed to be comparatively precise.

As much impurities existed in plasma in spite of the very minute level of 11-OHCS, it was not clear whether only 11-OHCS themselves were measured by the method or not. Then, source and emission spectra

of fluorescence radiated from plasma extracts of sheep, cattle and rats

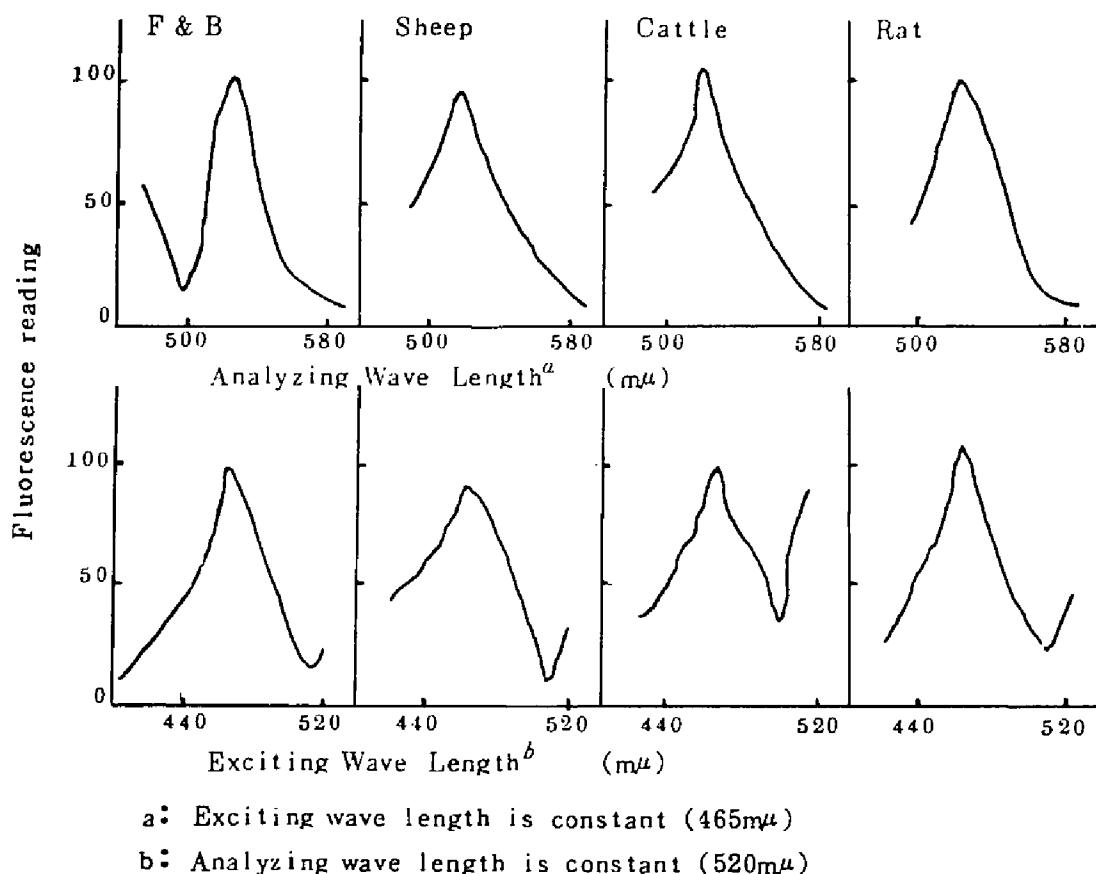
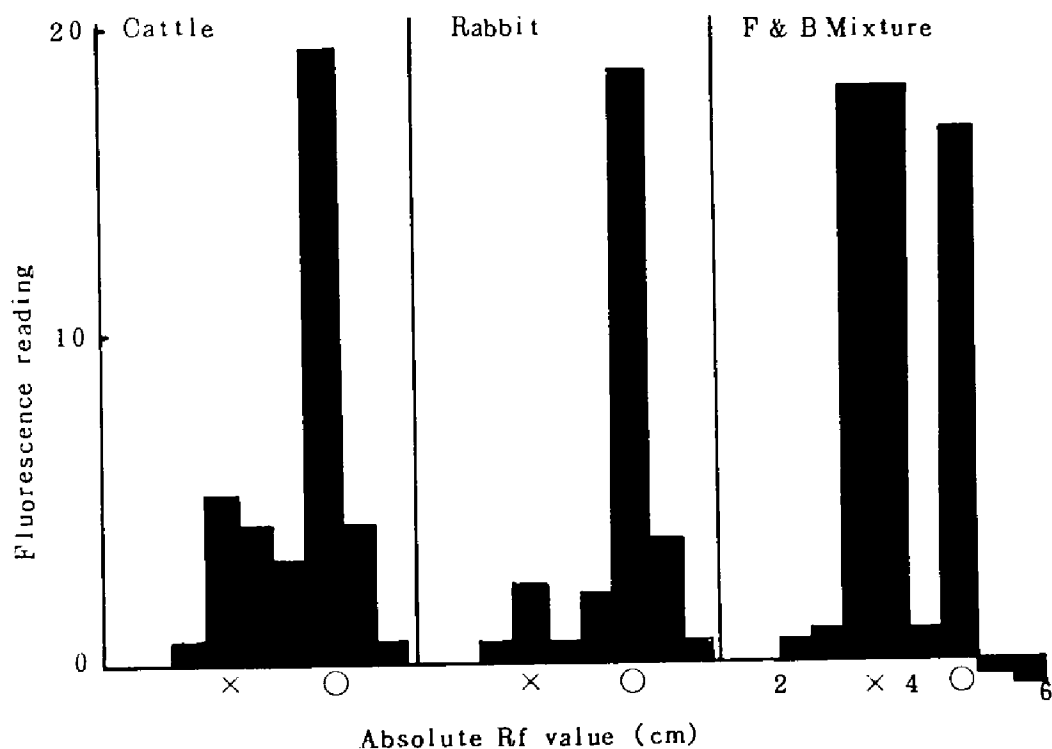


Fig. 11. Source and emission spectrum of fluorescence radiated from cortisol (F), corticosterone (B) and plasma extracts of sheep, cattle and rats

were investigated. The results are shown in Fig. 11. All samples showed the same curve as either cortisol or corticosterone standard and had a single peak at the wave length of 465 mμ or 520 mμ by the side of exciting or analyzing light, respectively. These results indicated that the impurities radiating fluorescence under the same conditions as 11-OHCS were almost perfectly ruled out from the present determination of 11-OHCS.

The separability of cortisol and corticosterone was examined on adrenal extracts of cattle and rabbits, and standard mixture of cortisol and

corticosterone (2 : 1). Fluorescence of the positions located on every 0.5 cm of width from the origin was measured. The good separation of



×: Shows the position of cortisol (F)
 ○: Shows the position of corticosterone (B)

Fig. 12. Thin layer chromatograms of cortisol (F), corticosterone (B) and adrenal extracts of cattle and rabbits

cortisol and corticosterone was appreciated by the chromatogram of standards as shown in Fig. 12. The F/B ratio of the standard was 1.98 and exactly equal to the mixing ratio (= 2).

Chapter II. Adrenal weights in various animals

The evaluation for adrenal weight as a way to know adrenocortical activity varied among investigators. DUYCK²⁸⁾ and SANZARI¹²⁵⁾ reported no significant correlation between adrenal weight and corticoid secretion rate in rats and dogs, respectively. On the other hand, SAYERS¹²⁸⁾ stated that adrenal weight is a good index of adrenocortical activity.

Suppose an organ occupies the part in body according to its need, it is considered that adrenal weight might be useful to evaluate the importance of adrenal glands. As the first step, adrenal weights were surveyed and compared among various animals.

1. Materials and Methods

Cattle (Japanese Black and Holstein), sheep (Japanese Corriedale), rabbits (Japanese native), domestic fowls (White Leghorn) and rats (Wistar strain) at the experimental farm in Kyoto University, were used in this investigation.

Japanese Black and Holstein were young fattening steers about eighteen months of age. The ages of other animals were about one to two years in sheep; sixteen weeks in domestic fowls; five months to two years in rabbits; one month to two years in rats.

Adrenal glands were taken out as soon as possible after slaughter of animals, and immediately trimmed and weighed.

2. Results and Discussion

Body weights and adrenal weights of various animals are shown in Table 5. Adrenal weight of the female rat was heavier than that of the male, but the sexual difference was not appreciated in Japanese Black Cattle and in rabbits.

SANZARI¹²⁵⁾ reported that a statistically significant correlation was found between body weight and adrenal weight. The adrenal weight per unit of body weight (AW/BW) has been often used for comparisons of the adrenal weight among animals whose body weights are different each other^{26,97)}. The AW/BW is shown in Table 6. The increasing order of AW/BW

Table 5. Body weights and adrenal weights in the various species of animals

Animal	Sex	Number	Body weight(kg)	Adrenal weight ¹⁾ (g)
Cattle Japanese Black Holstein	Female	6	4 2 8 ± 2 4 ²⁾	1 2.8 ± 2.0 ²⁾
	Steer	1 1	4 7 4 ± 3 2	1 3.7 ± 1.4
	Male	2	5 0 1 ± 1 8	1 3.7 ± 0.9
	Male	1 2	3 6 1 ± 1 5 0	1 3.6 ± 5.6
Sheep	Wether	9	3 3.5 ± 3.9	2 6.4 ± 0.4 8
Rabbit	Female	1 0	1.4 1 ± 0.2 3	0.2 3 2 ± 0.0 5 1
	Male	1 2	1.4 1 ± 0.2 1	0.2 3 4 ± 0.0 3 7
Domestic Fowl	Male	1 8	1.5 6 ± 0.0 9	0.1 3 8 ± 0.0 2 1
Rat	Female	2 4	0.1 9 5 ± 0.0 6 5	0.0 5 7 ± 0.0 1 9
	Male	2 5	0.2 7 4 ± 0.0 9 9	0.0 4 5 ± 0.0 1 3

1) The values are the combined weights of left and right adrenal glands

2) Mean ± standard deviation

was as follows; cattle < sheep < domestic fowls < rabbits < rats.

It was reported that AW/BW was 30.1 mg/kg (mean of six) in Japanese Beef Cattle¹⁶⁸⁾, 58 mg/kg (five) in sheep¹³⁵⁾, 176 mg/kg (ten)⁹⁷⁾, 90.5 mg/kg (twelve)¹⁶⁾ in the male white Leghorn, 76 mg/kg (six)¹⁶⁵⁾ in the laying hen, 107 to 136 mg/kg in the male rats^{26,57,93,123)} and 264 mg/kg (thirty five) in the female²⁶⁾. The present data coincided with these values.

Although AW/BW has been often adopted in the experiments dealt with a limited weight range, with a single species, or with a few closely related species, its manner cannot be useful over a large weight range²¹⁾. CHRISTIAN²¹⁾ recommended to take the ratio of logarithmic adrenal weight to logarithmic body weight.

From the point of view that the heat given off as the result of metabolism is directly proportional to body surface, i.e., metabolic body weight ($W^{3/4}$), it is supposed that the functional importance of

Table 6. A comparison of the adrenal weight per unit of body weight (AW/BW) and that of metabolic body weight (AW/MBW) among various species of animals

Animal	Sex	Adrenal weight	Adrenal weight
		Body weight (mg/kg)	Metabolic body weight ¹⁾ (mg/kg)
Cattle Japanese Black	Female	29.8 ± 3.9 ²⁾	13.55 ± 1.81 ²⁾
	Steer	29.2 ± 4.0	13.59 ± 1.71
	Male	27.3 ± 2.8	12.92 ± 1.22
	Holstein	39.0 ± 7.9	16.50 ± 3.19
Sheep	Wether	80.1 ± 18.8	19.15 ± 4.15
Rabbit	Female	16.72 ± 4.17	18.06 ± 4.19
	Male	16.90 ± 3.73	18.28 ± 3.64
Domestic Fowl	Male	88.7 ± 11.5	9.90 ± 1.33
Rat	Female	30.09 ± 4.77	19.48 ± 2.93
	Male	18.06 ± 5.73	12.50 ± 2.79

1) Metabolic body weight is body weight to the three-quarters power

2) Mean ± standard deviation

adrenal glands related to metabolism could be evaluated through adrenal weight per unit of the metabolic body weight (AW/MBW). There was no significant difference in the AW/MBW among animals except that the AW/MBW of domestic fowls was significantly small.

If either body weight or metabolic body weight was more closely related to the adrenal weight, the variability in values of the adrenal weight divided by the more closely related weight should be smaller than the other. Nevertheless, their co-efficients of variation were nearly equal; $18.8 \pm 6.8\%$ in the AW/BW and $17.0 \pm 4.8\%$ in the AW/MBW. It is suggested that both indicators are equally valuable as a measure, if any, which shows an importance of adrenal glands in body.

As a whole, adrenal glands in ruminants were smaller than that in non-ruminants in regard to the degree of occupancy, but the former were functionally as important as the latter.

Chapter III. 11-OHCS levels in both blood plasma and adrenal tissue of various animals

It has been stated that glucocorticoid levels in plasma^{73,111)} and in adrenal tissue⁵⁷⁾ are the good criterions of the adrenocortical activity. Although many investigators have determined glucocorticoid levels in plasma of various animals^{7,26,73,97,106,123,124,150,152,163)}, there exist some questionable problems in their methods. No report concerning plasma glucocorticoid levels under the same conditions through various animals is available yet. Levels of glucocorticoids in adrenal glands have not been reported in most of domestic animals.

It has been known that the majority of glucocorticoids in both plasma and adrenal glands is either cortisol or corticosterone (or a mixture of both) in all species, but the relative amounts of cortisol and corticosterone vary widely among animal species^{19,45)}. Moreover, it was described that there might be difference between the action of cortisol and that of corticosterone⁴²⁾. From such reasons, it was necessary to consider not only the total level of 11-OHCS, but also F/B ratio in plasma and adrenal glands.

11-OHCS levels and F/B ratios in both plasma and adrenal glands were compared among various animals.

1. Materials and Methods

The used animals, the bleeding technique and the recipe for blood plasma and adrenal glands were described previously (Chapter I). The samples were carefully collected under the conditions to give "resting level" of 11-OHCS.

11-OHCS levels and F/B ratios in plasma and adrenal tissue were determined according to the methods obtained in the previous study (Chapter I).

2. Results and Discussion

The levels of 11-OHCS in both plasma and adrenal tissue are shown in Table 7. The levels of 11-OHCS in plasma of cattle, sheep, goats

Table 7 A comparison of 11-OHCS levels in plasma and adrenal tissues among various species of animals

Animal	Sex	Plasma 11-OHCS level ($\mu\text{g}/100\text{ ml}$)	Adrenal 11-OHCS level ($\mu\text{g}/100\text{ mg}$)
Cattle ^{a)}	Female	— ¹⁾	$4.29 \pm 1.73 (6)^{2)}$
	Steer	$2.94 \pm 0.84 (6)^{2)}$	$3.07 \pm 0.58 (12)$
	Male	$2.17 \pm 0.64 (4)$	$2.83 \pm 1.23 (4)$
	Holstein	$3.43 \pm 0.95 (15)$	$3.72 \pm 1.14 (10)$
Sheep ^{a)}	Wether	$3.20 \pm 1.16 (16)$	$3.11 \pm 2.48 (6)$
Goat ^{a)}	Female	$3.33 \pm 0.83 (2)$	—
	Male	$2.11 \pm 0.11 (2)$	—
Rabbit ^{b)}	Female	$6.23 \pm 4.08 (13)$	$2.30 \pm 1.19 (7)$
	Male	$7.46 \pm 2.72 (18)$	$1.97 \pm 0.62 (6)$
Domestic Fowl ^{b)}	Male	$2.18 \pm 0.19 (6)$	$0.04 \pm 0.02 (4)$
Rat ^{b)}	Female	$4.53 \pm 3.30 (22)$	$0.70 \pm 0.37 (4)$
	Male	$3.13 \pm 1.82 (24)$	$0.70 \pm 0.23 (4)$

1) No datum available

2) Mean $\pm$ standard deviation; a number in parentheses shows the number of samples

a) 11-OHCS levels of the animal are presented as cortisol;

b) 11-OHCS levels of the animal are presented as corticosterone

and domestic fowls were lower than those of rabbits. The mean levels of female and male rabbits were $6.23\ \mu\text{g}/100\text{ ml}$ and $7.46\ \mu\text{g}/100\text{ ml}$, respectively, and evidently lower than those of rats. And the female rats were higher than the male.

The levels of 11-OHCS in adrenal glands of cattle and sheep were higher than those of domestic fowls, rabbits and rats as shown in Table 7. CRITCHLOW *et al.*²⁶⁾ reported that 11-OHCS levels in adrenal glands of rats were $1.96 \pm 0.27\ \mu\text{g}/100\text{ mg}$ in female and $0.89 \pm 0.15\ \mu\text{g}/100\text{ mg}$ in male. These values were in agreement with the present data.

On the results in Table 7, adrenal 11-OHCS levels were higher in ruminants than in non-ruminants, while plasma 11-OHCS levels were the reverse of the adrenal. Cattle which the adrenal samples were taken from, however, had been transported 60 km from the farm to the slaughter house and fasted for about two days. And the sheep had been transferred from their barn into the dissecting room and tethered in the room for a few hours. REID *et al.*¹¹⁷⁾ reported that transportation influenced cortisol level in plasma of sheep. BARRETT *et al.*⁵⁾ reported that rats transferred to laboratory for experiment had high plasma and adrenal corticosterone levels. From these facts, the adrenal 11-OHCS levels found in cattle and sheep may not represent the "resting level". However, it is not well known to what degree such factors would influence on adrenal 11-OHCS level in ruminants. Therefore, further investigation will be necessary in order to discuss on the difference of the levels among species.

It was reported that plasma glucocorticoid levels were about $1.5 \mu\text{g}/100 \text{ ml}$ in sheep^{73,124)}, and approximately $10 \mu\text{g}/100 \text{ ml}$ in the male rats and $30 \mu\text{g}/100 \text{ ml}$ in the female^{26,39)}. The present levels of both rats and sheep were somewhat higher than the results found in the literatures^{7,26,39,73,106,124)}. On the contrary, the level of dairy cattle was lower than the result reported by ROBERTSON *et al.*¹²¹⁾ and VENKATASESHU and ESTERGREEN¹⁶¹⁾, but higher than the result by THOMPSON *et al.*¹⁵⁰⁾. And, WILLETT *et al.*¹⁶³⁾ reported the similar level to the present result in dairy cattle.

As the reasons brought about these differences, environmental conditions, and sex, breed, age, etc. of the experimental animal could be considered. Such influences on plasma 11-OHCS level, however, is not sufficiently understood. Then, further investigation will be carried out later on.

Plasma glucocorticoid levels in some other animals have been reported; 10 to $24 \mu\text{g}/100 \text{ ml}$ in humans^{15,109,137,138)}, $40 \pm 0.8 \mu\text{g}/100 \text{ ml}$ in monkey¹³⁷⁾, $34.0 \pm 0.7 \mu\text{g}/100 \text{ ml}$ in guinea pigs¹³⁷⁾, $18.4 \pm 5.9 \mu\text{g}/100 \text{ ml}$ in pigs¹⁵²⁾ and 5 to $10 \mu\text{g}/100 \text{ ml}$ in mice⁷¹⁾.

As shown in Table 8, F/B ratio in plasma was over 3.0 in ruminants; about 2.0 in rabbits; and under 0.5 in domestic fowls and rats. However, the ratios in adrenal tissue were under 0.5 in most of the tested animals, except for the higher ratio in sheep; 2.9. F/B ratio in plasma was

Table 8. A comparison of the ratios of cortisol to corticosterone (F/B ratio) in plasma and adrenal tissues among various species of animals

Animal	Sex	F/B ratio	
		in plasma	in adrenal
Cattle Japanese Black	Female	— 1)	0.4
	Steer	3.6	0.2
	Male	2.3	0.3
	Holstein	6.5	0.3
Sheep	Wether	3.8	2.9
Goat	Female	10.0	—
	Male	3.6	—
Rabbit	Female	2.3	0.3
	Male	1.9	0.2
Domestic Fowl	Male	0.4	0.1
Rat	Female	0.2	0.04
	Male	0.4	0.00

1) No datum available

different from that in adrenal tissue even in the same animal.

The present results of F/B ratio in adrenal tissue were similar to the data described on beef cattle¹⁷⁰⁾, rats⁵⁷⁾ and other animals⁴⁵⁾, respectively. Although the data regarding F/B ratio in plasma had rarely been available, it was recently reported that the ratios in plasma of Holstein cattle were 4.0 ± 1.2 ¹⁶¹⁾ and 2.4 ¹⁶³⁾. These values coincided with the present results.

According to the results in Tables 7 and 8, ruminants had lower level of 11-OHCS ($\leq 4 \mu\text{g}/100 \text{ ml}$) and higher F/B ratio (≥ 3) in plasma than non-ruminants. In regard to the low level of glucocorticoids in plasma of ruminants, three possibilities can be suggested: 1) The higher sensitivity of ruminants to the corticoids as LINDNER⁷³⁾ has already

pointed out. 2) The existence of a specific steroid in plasma of ruminants. 3) The quickness and intensity in response of adrenal cortex in ruminants to many stressors.

Moreover, cortisol was predominant in plasma of ruminants. This might be an adaptative symptom to the metabolism characteristic of ruminants, as it is well known that the corticoid has the highest gluconeogenetic activity.

Chapter IV. Effect of season, age and sex on plasma 11-OHCS level

Adrenal weights and 11-OHCS levels in plasma and adrenal tissue of several domestic and laboratory animals, were presented previously together with F/B ratios (Chapters II and III). For the proper evaluation of these results, possible effects of season, age and sex on these parameters should be taken into consideration. Among a few available reports, PATERSON¹⁰⁵⁾ described that plasma 11-OHCS levels in cattle were influenced by season, and WATANABE¹⁶²⁾ reported that the similar effect existed in humans. Regarding the effect of age, SHAW *et al.*¹³⁴⁾ reported that plasma 11-OHCS levels of calves, 3 to 8 months old, were similar to those of adult cows. The sexual difference in plasma 11-OHCS level was apparent in mature rats^{26,39)}, but that in sheep or in cattle has not been known.

The purpose of this study is to elucidate effects of season, age and sex on plasma 11-OHCS levels of cattle, sheep and rats.

1. Materials and Methods

The breed and the number of animals are presented in Table 9. Cattle were fed at Tottori Livestock Breeding Station** and Department of Animal Industry, Chugoku Agricultural Experimental Station**. Sheep were fed at Chugoku Livestock Breeding Station**. Rats were fed at the laboratory, in Kyoto University and they were moved into individual cages 10 days before starting each experiment. The animals

Table 9. The breed and the number of animals

Species	Breed	Number (heads)		
		Male	Female	Total
Cattle	Japanese Black	67	140	207
Sheep	Japanese Corriedale	54	71	125
Rat	Wistar	24	22	46

consisted of both sexes of various ages.

The seasons tested were early summer (June, 1969) and late winter (February, 1970).

Bleeding technique and recipe for blood plasma were as described previously (Chapter I). According to ZIMMERMAN *et al.*¹⁷¹⁾, it took several minutes for rats to show the response of plasma corticosterone level after exposure to a stressor. The time required for bleeding was less than one minute in this study. Therefore, the blood samples have presumably had 'resting level' of plasma 11-OHCS.

Blood was taken under the conditions as stress-free as possible late in the morning. The blood sample was centrifuged and the supernatant plasma was stored at -20°C . The determination of 11-OHCS in plasma was carried out by the method described previously (Chapter I).

** : All these stations belong to Ministry of Agriculture and Forestry of Japan.

Table 10. Sexual and seasonal differences in plasma 11-OHCS levels of cattle, sheep and rats

Species	Season	Plasma 11-OHCS level ($\mu\text{g}/100\text{ml}$)	
		Male	Female
Cattle	Summer	$147^a \pm 0.49(30)^*$	$181^a \pm 0.85(69)$
	Winter	$166 \pm 0.76(37)$	$177 \pm 0.94(71)$
Sheep	Summer	$305 \pm 1.06(30)$	$278 \pm 0.96(34)$
	Winter	$327 \pm 1.40(24)$	$302 \pm 1.19(37)$
Rat	Summer	$2698 \pm 2129(14)$	$3972 \pm 3019(12)$
	Winter	$3695^b \pm 1211(10)$	$5203^b \pm 3660(10)$

a and b : Means having a common letter in their superscripts are statistically different ($P < 0.05$)

* : Mean $\pm$ standard deviation; The number of observations is indicated in parentheses

2. Results and Discussion

Plasma 11-OHCS levels of all animals in early summer and late winter are shown in Table 10. It was also observed in this study that plasma 11-OHCS levels of cattle and sheep were quite low as compared to those of rats, regardless of seasons and sexes.

Plasma 11-OHCS levels of cattle and sheep in winter were similar to those in summer, while the levels of rats in winter were higher than those in summer though the difference was not significant.

In cattle and rats, plasma 11-OHCS levels of the female seemed to be higher than those of the male. The sexual difference was significant in the case of cattle in summer ($P < 0.01$) and rats in winter ($P < 0.05$). On the other hand, the difference was obscure in sheep.

Values of plasma 11-OHCS in Table 10 were further classified into several groups according to the age as shown in Tables 11, 12 and 13, to facilitate the analysis of possible effects of sex and season.

Plasma 11-OHCS levels of male and female cattle seven to twelve months old were higher in winter than those in summer, but this seasonal difference was significant only in the male ($P < 0.01$). The seasonal effects in either younger or older ages were not significant (Table 11). In sheep, age did not affect the sexual and seasonal difference in plasma 11-OHCS levels except that the levels in the females older than 2 years decreased with age (Table 12).

In rats, data obtained were limited. Only a conclusion may be that the plasma 11-OHCS levels in this species are readily affected by the season, high in winter and low in summer. Effect of sex was obvious only in the adult (Table 13). CRITCHLOW *et al.*²⁶⁾ reported the similar effects in rats.

In general, plasma 11-OHCS levels in ruminants were less susceptible to season than those in rats. However, it was interesting that plasma 11-OHCS levels in cattle 7 to 12 months old were sensitively affected by season. This age range coincides with that in which calves are susceptible to Kiriyo disease¹⁴⁵⁾, and hence adrenal dysfunction could be involved in its etiology.

Table 11. The influence of age on sexual and seasonal differences in plasma 11-OHCS levels of cattle

Sex	Plasma 11-OHCS level ($\mu\text{g}/100\text{ ml}$)					
	Under 6 months		7-12 months		Over 13 months	
	Summer	Winter	Summer	Winter	Summer	Winter
Male	$1.50 \pm 0.30(10)^*$	$1.78 \pm 0.85(19)$	$1.50 \pm 0.66(11)^a$	$2.18 \pm 1.05(6)^a$	$1.41 \pm 0.44(9)$	$1.45 \pm 0.70(12)$
Female	$2.46 \pm 1.18(12)$	$1.26 \pm 0.41(11)$	$1.58 \pm 0.51(4)$	$2.71 \pm 1.29(10)^b$	$1.67 \pm 0.71(53)$	$1.73 \pm 0.83(50)^b$

^a and ^b: Means having a common letter in their superscripts are statistically different ($P < 0.01$)

* : Mean $\pm$ standard deviation; The number of observations is indicated in parentheses

Table 12. The influence of age on sexual and seasonal differences in plasma 11-OHCS levels of sheep

Sex	Plasma 11-OHCS level ($\mu\text{g}/100\text{ ml}$)					
	2-4 months		11-17 months		Over 2 years	
	Summer	Winter	Summer	Winter	Summer	Winter
Male	$2.60 \pm 0.79(12)$	—	$3.29 \pm 0.88(6)$	$3.48 \pm 1.62(14)$	$3.37 \pm 1.27(12)$	$2.97 \pm 1.03(10)$
Female	$2.67 \pm 0.79(11)$	—	$3.28 \pm 1.00(6)$	$3.69 \pm 1.37(13)^a$	$2.69 \pm 1.04(17)$	$2.66 \pm 0.91(24)^a$

^a: Means having a common letter in their superscripts are statistically different ($P < 0.01$)

Table 13. The influence of age on sexual and seasonal differences in plasma 11-OHCS levels of rats

Age	Season	Plasma 11-OHCS level ($\mu\text{g}/100\text{ml}$)	
		Male	Female
Old	Winter	4 1.7(2) ^{a)}	1 8.1(2)
Middle	Summer	2 5.2(5)	4 5.1(5)
	Winter	3 2.3(3)	5 4.4(3)
Young	Summer	2 2.0(6)	3 4.3(5)
	Winter	3 7.8(5)	6 4.1(5)
Suckling	Summer	3 9.9(3)	4 0.0(2)

a): Number in the parentheses shows the number of samples

Chapter V. Effect of physical and psychological stresses on plasma 11-OHCS level

Seasonal variations in plasma 11-OHCS levels were investigated previously (Chapter IV). It was suggested that the level in cattle and sheep might be more stable than in rats as described by KNIGGE⁶⁸⁾, indicating that adrenal cortex of ruminants might respond less sensitively to a stressor than that of rats. There are some evidences^{116,131)} to support the inference.

In this study, the responses of plasma 11-OHCS level were investigated by exposing the selected species of animals to some stressors. Meteorological stressors, *i.e.*, 'cold' and 'hot' temperatures, and transportation were selected as physical and psychological stressors according to REID^{116,117)}.

1. Materials and Methods

Young fattening steers about 16 months old (Japanese Black), adult wethers (Japanese Corriedale) and adult male rabbits (Japanese native) and rats (Wistar strain) at the experimental farm in Kyoto University were used.

This study consisted of 3 experiments. The treatment of each experiment was as follows; Exp. I ('hot' stress): sheep and rats were moved from outdoor environment into a hot room of phytotron at about 6 A.M. The temperature was increased 16 – 21°C by the transfer (Table 14). Exp. II ('cold' stress): sheep and rats kept in the hot room for five days were moved back to the outdoor environment at 1 and 6 – 7 A.M., respectively. The temperature was decreased 15 – 20°C by the second transfer. These two experiments were performed in the period from February 6 to March 18, in 1970 in Kyoto. Blood samples of sheep (4 heads) were taken from each animal at 0, 1, 2, 4 and 8 hours after the move. In rats (8 heads), the samples were taken from different animals allotted to each time, *i.e.*, 0, 1/2, 1 and 2 hours.

Exp. III (psychological stress): cattle, sheep and rabbits were transported by a truck for 120, 90 and 10 minutes, respectively. Blood samples were taken from each animal at 0, 1, 2, 4 and 8 hours after the beginning

Table 14. Conditions of Experiments I and II

Animal	Experiment I ('hot' stress)			Experiment II ('cold' stress)		
	Date ^{a)}	Time at the start of treatment	Difference of the temperature (°C)	Date ^{a)}	Time at the start of treatment	Difference of the temperature (°C)
Sheep	2 6	a.m. 5:30	21(-1-20) ^{b)}	2 12	a.m. 1:00	15(20-5) ^{b)}
	3 2	a.m. 6:30	21(-2-19)	3 7	a.m. 1:00	19(20-1)
Rat	2 16	a.m. 6:30	18(4-20)	2 26	a.m. 7:00	20(20-0)
	3 7	a.m. 6:00	19(1-20)	3 18	a.m. 6:00	18(20-2)

a) These experiments were carried out in Kyoto in 1970

b) The numbers in parentheses show the temperatures of outdoors and the room of phytotron

of transportation in sheep (4 heads). In rabbits blood was taken from all animals (12 heads) 10 days before the exposure to the stressor to determine the initial level of plasma 11-OHCS. After the exposure, the samples were taken from different animals allotted to each time, i.e., 1/2, 1, 2 and 4 hours. In cattle (4 heads) it was also made from each animal at one day before, and 2 and 24 hours after the beginning of transportation.

The bleeding technique and the method for determination of plasma 11-OHCS were described previously (Chapter I).

2. Results

As shown in Fig. 13, plasma 11-OHCS levels in sheep somewhat increased at 4 hours after the exposure to the stressor, but decreased beneath the initial value at 8 hours. In rats, the levels increased rapidly and significantly ($P < 0.01$), but returned to the initial value within 1 hour after the exposure.

Plasma 11-OHCS levels in sheep and rats responded to the 'cold' stress as shown in Fig. 14. The changes in sheep after the exposure to the stressor were not significant, if any. However, the levels in

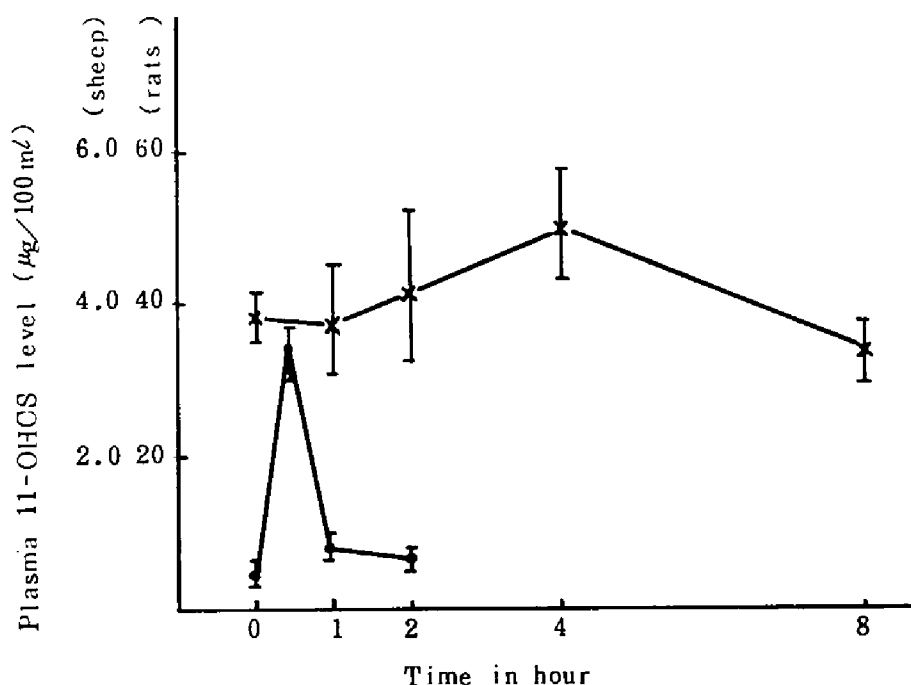


Fig. 13 Influence of 'hot' stress on plasma 11-OHCS level in sheep and rats (Exp. I)

Each mark shows mean plasma 11-OHCS level and standard deviation (cross (×)-sheep, solid circle (●)-rats)

rats increased rapidly and significantly ($P < 0.05$) after the exposure, and it remained high level even after two hours.

After transportation the levels in cattle and sheep increased twice the initial value. The levels in rabbits increased three times the initial regardless of the shorter duration of transportation (Fig. 15).

3 Discussion

In general, the actions of glucocorticoids may be divided into two main classes: one is to help or protect the animal body from stresses by increasing the resisting ability, and the other is to regulate the metabolic pattern⁴¹⁾.

It was obtained the results that plasma 11-OHCS levels in sheep were not changed significantly by meteorological stressors, but those in

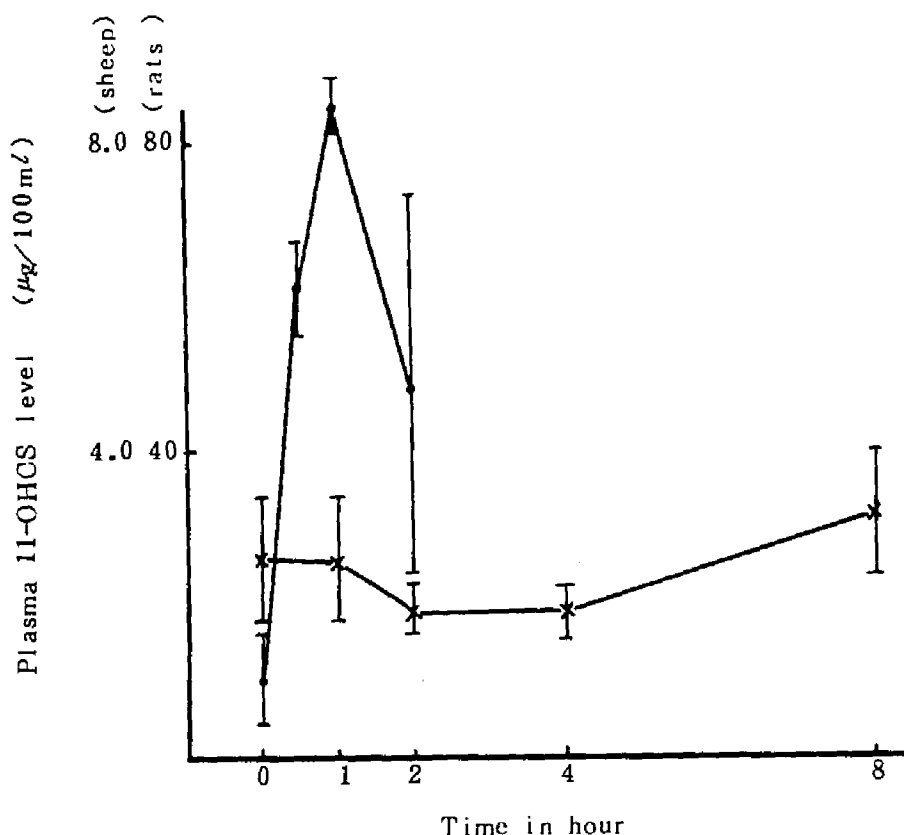


Fig. 14. Influence of 'cold' stress on plasma 11-OHCS level in sheep and rats (Exp. II)

Each mark shows mean plasma 11-OHCS level and standard deviation (cross (X)-sheep, solid circle (●)-rats)

rats were increased rapidly and significantly. REID¹¹⁶⁾ reported that plasma cortisol level in sheep was not altered by exposure to cold, even in combination with other types of stressors; undernutrition and pregnancy. On the other hand, acute exposure to cold caused a transient rise of adrenocortical activity in rats, though the activity was indirectly evaluated by adrenal ascorbic acid depletion test^{131,166)}. And in humans a cold exposure increased the adrenocortical activity¹⁶⁴⁾.

Plasma 11-OHCS levels increased rapidly in all the animals subjected to transportation (Fig. 15). The response in rabbits was more remarkable

than that in either sheep or cattle. REID *et al.*¹¹⁷⁾ reported that serum cortisol level in sheep was increased by transportation. MESCHAKS⁹⁰⁾ reported that urinary neutral steroids increased in the bull following 15 — 40 km transportation. It is obvious that transportation of long duration might be stressful in physical means, but effects of transportation of short duration should be investigated from psychological viewpoints. REID *et al.*¹¹⁷⁾ and REID¹¹⁶⁾ listed the short transportation as a psychological stressor.

So far as plasma 11-OHCS are concerned, it is suggested that ruminants are less sensitive to a physical and a psychological stress than non-ruminants. The results also indicate that a psychological stress would cause rather trouble in the practical husbandry of sheep than a physical stress.

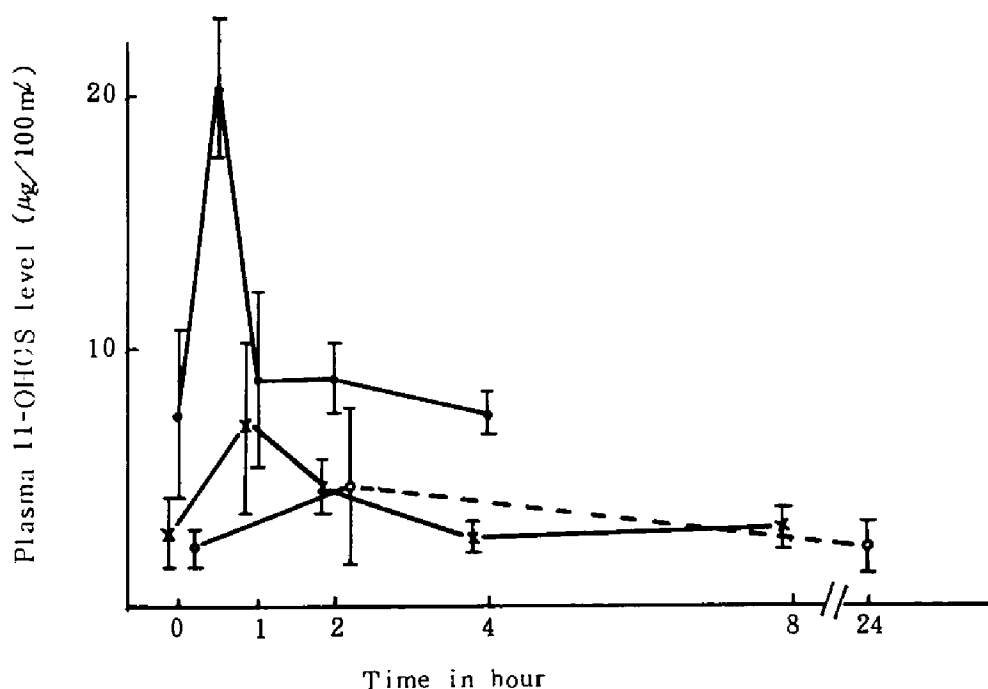


Fig. 15. Influence of transportation on plasma 11-OHCS level in cattle, sheep and rabbits (Exp. III)

Each mark shows mean plasma 11-OHCS level and standard deviation (cross (X)-sheep; solid circle (●)-rabbits, open circle (○)-cattle)

Chapter VI. Effect of fasting on plasma 11-OHCS level

Many investigators reported that plasma cortisol level was not altered during fasting in humans^{59,66,70,129}). Data on plasma glucocorticoid measurements in rodents during fasting are sparse. Chronic starvation (4 - 14 days) failed to elicit any sign of adrenocortical stimulation in rabbits¹⁴). A 24 hours fasting in the rat produced an increase in plasma corticosterone levels, but this had been generally ascribed to nonspecific stress³⁸). The effect of prolonged fasting on plasma corticosterone level is not known in rats. On the other hand, OTSUKA and OMORI¹⁰¹) suggested that adrenal cortex of goats might be activated by fasting, based on the information regarding peripheral blood eosinophils count and adrenal weight.

These results and suggestion seemed to support the hypothesis that adrenocortical function of ruminants might be of particular importance in relation to nutrition, compared with that of nonruminants.

In this study, the responses of plasma 11-OHCS levels and adrenal weights to fasting were observed to compare an importance of the adrenocortical function regarding the regulation of intermediary metabolism in ruminants with that in rodents.

1. Materials and Methods

The animals used in this study were the same as described previously (Chapter V). Each animal was kept individually in a metallic cage. Water was given *ad libitum* during fasting.

Plasma samples were taken from each animal (4 heads) at 0, 1, 2, 4, 10, 16, 20, 24, 28, 32, 36 and 40th day after removal of feeds in sheep. It was also made from the different animals allotted to each particular day; 0, 1, 2, 3, 4, 6, 10 and 14th day in rabbits (18 heads) and ; 0, 1, 2, 3, 4 and 7th day in rats (21 heads) after the removal. Bleeding technique was described previously (Chapter I). Body weight was measured every at bleeding.

Plasma 11-OHCS levels were determined in the four rats fasted for 5 days and in the three fed controls. All these rats were decapitated at the same time.

The determination of plasma 11-OHCS level was carried out by the method described previously (Chapter I).

2. Results

Plasma 11-OHCS levels and body weights during fasting in sheep, rabbits and rats are shown in Figs. 16, 17 and 18, respectively. The mean initial body weights with standard deviation were 46 ± 4.4 kg in sheep, 2.83 ± 0.13 kg in rabbits and 341 ± 86 g in rats. In the figures, the body weight was shown as the percentage to each initial weight. Initial levels of plasma 11-OHCS were in agreement with those in the previous experiment (Chapter III) in either sheep or rabbits, whereas the levels were not in rats. The reason for this discrepancy was not obvious, but it might result from the different meteorology, season, etc. (Chapter III).

Plasma 11-OHCS levels in sheep declined slightly after first day of fasting, but increased promptly and reached a plateau within 10 days after the removal of feeds. At tenth day, the level was significantly higher than the initial ($P < 0.05$). The levels increased moreover from the primary to the secondary plateau at 20 to 24th day after the removal. Prior to death, however, plasma 11-OHCS levels decreased suddenly (Fig. 16). Three sheep died after 39, 42 and 43 days of fasting. Then, fasting was terminated, and one sheep was re-fed to recover.

Body weight decreased rapidly and acceleratively for 10 days from the beginning of fasting and reached 76.5 % of the initial at that time, but the weight decreased slowly and constantly thereafter in sheep (Fig. 16).

Plasma 11-OHCS levels in rabbits responded to fasting as shown in Fig. 17. The levels were lower between 1 and 6th day after the removal of feeds than the initial, but became higher at 10th day. The levels declined after 14 days of fasting again. These changes were not statistically significant. This experiment was ceased when one rabbit died between 10 and 14th day after the removal.

Body weight decreased gradually and reached 79.4 % of the initial weight at 10th day after the removal of feeds in rabbits (Fig. 17)

As shown in Fig. 18, the mean plasma 11-OHCS level in rats seemed

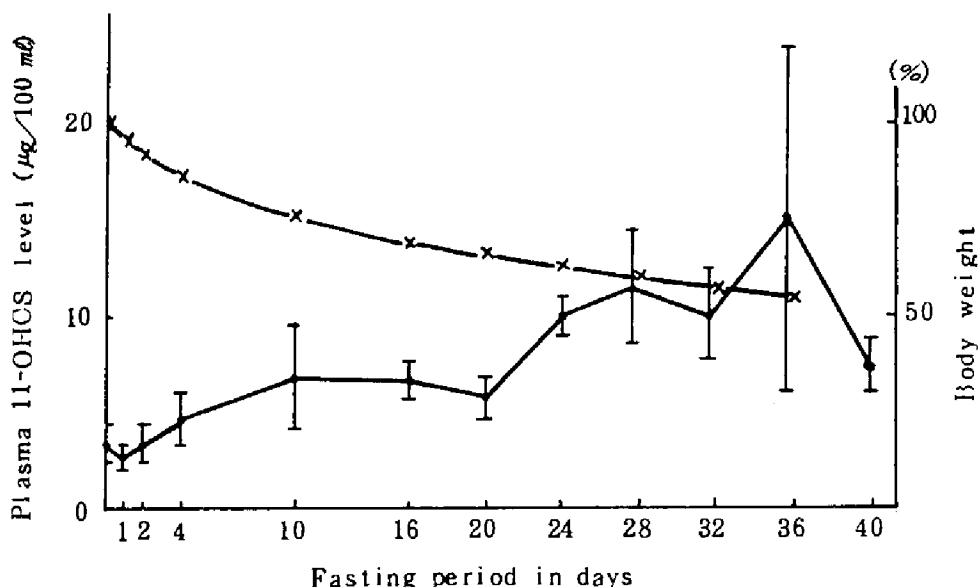


Fig. 16. Influence of fasting on plasma 11-OHCS level and body weight in sheep

Solid circles (●) represent mean plasma 11-OHCS level with the standard deviation and crosses (×) represent the mean percent of body weight to the initial.

to increase after first day of fasting and remained higher level than the initial thereafter. One rat died at 6th day after the removal of feeds. The changes of plasma 11-OHCS levels by fasting were not statistically significant because of the large variation.

Body weight decreased rapidly and acceleratively for 3 days from the beginning of fasting, but gradually thereafter in rats. The weight at third day was 78.4 % of the initial (Fig.18).

The further experiment was carried out to elucidate whether plasma 11-OHCS levels were affected by fasting in rats or not under the conditions without the effect of meteorology, season, etc.. Body weight decreased to 78.8 % of the initial after 5 days of fasting. The mean plasma 11-OHCS levels were $46.4 \pm 33.4 \mu\text{g}$ (S.D.) per 100 ml in the fasted group and $31.5 \pm 10.2 \mu\text{g}$ (S.D.) per 100 ml in the fed. The difference between the two group was not significant.

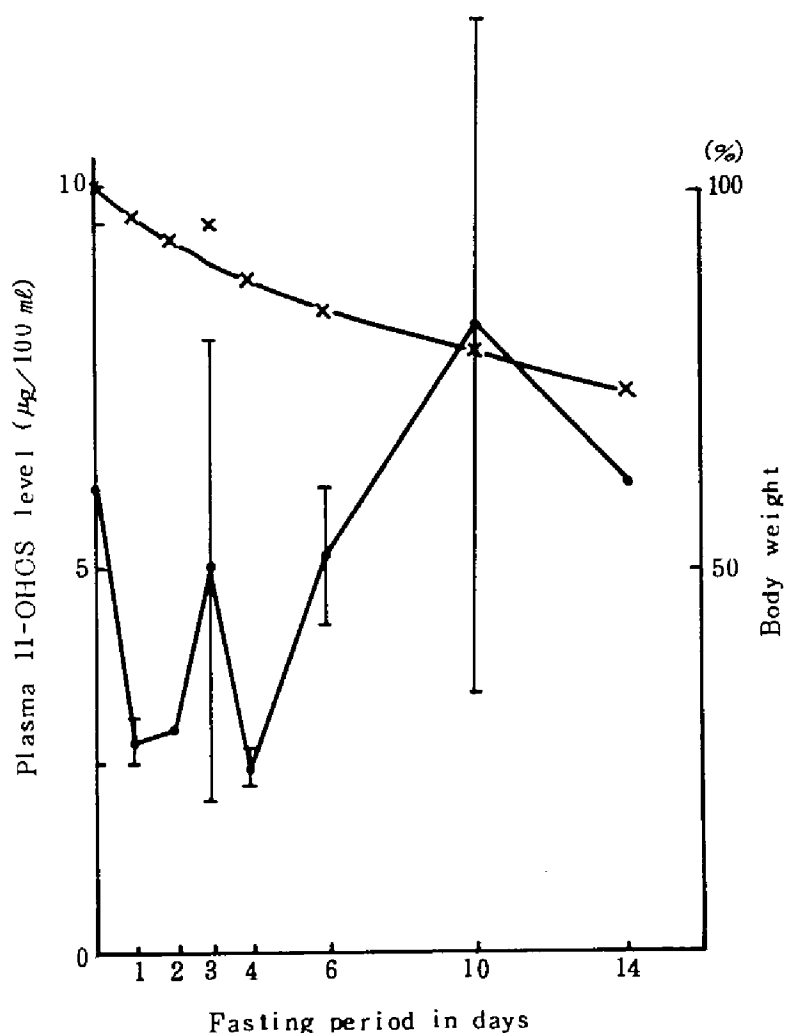


Fig. 17 Influence of fasting on plasma 11-OHCS level and body weight in rabbits

Solid circles (●) represent mean plasma 11-OHCS level with the standard deviation and crosses (×) represent the mean percent of body weight to the initial

Weights of adrenal glands, liver and kidney in sheep died of fasting are shown in Table 15. Their normal relative weights were 76 mg/kg, 12.2 and 2.31 g/kg, respectively, according to MIYAZAKI⁹²⁾. Adrenal weight in the fasted sheep seemed to be heavier than the normal, while the liver and kidney weights were comparable to it. On the other hand, the relative adrenal weights in rats were not changed significantly by fasting, as shown in Table 16.

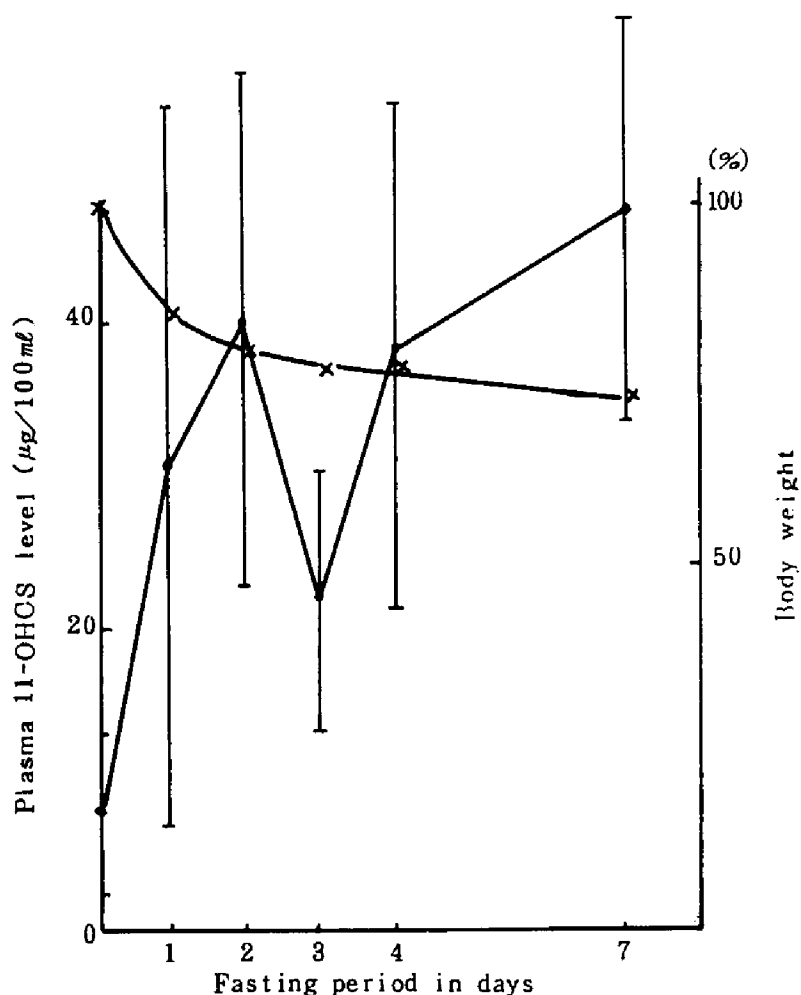


Fig. 18 Influence of fasting on plasma 11-OHCS level and body weight in rats

Solid circles (●) represent mean plasma 11-OHCS level with the standard deviation and crosses (×) represent the mean percent of body weight to the initial

3 Discussion

In general, the amount of carbohydrate which is present in a body is very small; occurring as much less than 1% of the body at any given moment. But, it is constantly being formed and broken down in metabolism and thus performs a multitude of vital functions⁸³⁾. A great amount of glucose was absorbed from the gut in most of animals, while only little part of carbohydrate in feeds was absorbed as glucose in ruminants^{3,52)}.

And that, glucose utilization expressed as a function of metabolic body size ($W^{3/4}$) in ruminants was comparable to that in non-ruminants according to BAXTER *et al.*¹⁰⁾. Therefore, one could have a hypothesis that gluconeogenetic action of glucocorticoids was of particular importance in ruminants.

Gluconeogenesis, the process by which non-carbohydrate precursors are converted to glucose, is of especial importance during fasting or when low carbohydrate diets are consumed¹⁵⁵⁾. Then, the responses of plasma 11-OHCS levels to fasting were investigated in sheep, rabbits and rats.

During fasting, body weight decreased rapidly and acceleratively from 100 to 80 %, but slowly and constantly thereafter. This seemed to indicate that an animal had reached to 'fasting condition', when body weight declined under 80 % of the initial, and that feeds in gut of the animal had been absorbed almost completely at the time¹²⁶⁾. At the 'fasting condition', plasma 11-OHCS levels in sheep responded significantly ($P < 0.05$) to fasting, whereas the levels in rabbits and rats not significantly. The result in sheep was in agreement with that in goats reported by OTSUKA¹⁰¹⁾. On the other hand, no response of plasma glucocorticoid level to fasting was described in humans^{59,66,70,129)} and rabbits¹⁴⁾.

It was reported that the kidney of sheep fasted for 10 to 14 days synthesized more glucose *in vitro* from amino acids, especially alanine and serine, than that of the fed animal¹²⁷⁾. Gluconeogenesis from various substrates was also enhanced by fasting in rats¹³⁰⁾. Moreover, it has been demonstrated that glucocorticoids stimulate the incorporation of L-alanine- $U-C^{14}$ into glucose^{99,118)} and other gluconeogenesis^{30,41,51,53,99,157)}. The stimulated glucose synthesis during fasting may be mediated by the adrenocortical function in ruminants, while it may be mediated by other factors in non-ruminants.

As a conclusion, it is suggested that adrenal cortex of sheep responds more sensitively to the increased requirement of glucose synthesis than that of either rats or rabbits. This relation was the reverse of a species difference in the response to meteorological and transportation stressors (Chapter V). These results seem to support the hypothesis that gluconeogenetic action of glucocorticoids is more important in

ruminants than the hormonal action to protect the body from stresses. This problem will be also discussed in the following chapter (Chapter VII).

Table 15. The wet weight of adrenal glands, liver and kidney in the sheep died of fasting and in those normally fed

Item	Number	Relative weight ^{a)}		
		Adrenal glands (mg/kg)	Liver (g/kg)	Kidney (g/kg)
Fasted sheep	3	116±38 ^{b)}	14.1±4.6	2.33±0.47
Normal ^{c)}	12	76	12.2	2.31

a): Relative weight indicates the weight of organ per kg of initial body weight

b): Mean±standard deviation

c): These data were quoted from a paper by MIYAZAKI, A.; Jap. J. Zootech. Sci., 38 (1967) 527-536

Table 16. The wet weight of adrenal glands in fasted rats

Period fasted (days)	0	1	2	3	4	7
Absolute weight (mg)	44.0±12.8	51.6±8.2	47.6±7.5	53.7±12.3	51.7±10.8	44.0±2.7
Relative weight (mg/kg)	128.1±6.4 ^{a)}	147.0±12.9	138.2±14.8	152.2±16.2	144.9±19.0	133.8±22.7

a): Mean and standard deviation of relative weight which is the adrenal weight in mg per kg of initial body weight

Chapter VII. Role of glucocorticoids on glucose metabolism in fasting animals

The results in the previous experiment suggested that adrenal cortex of sheep might respond more sensitively to fasting than that of either rats or rabbits (Chapter VI). In order to know reasons of these difference between sheep and other animals, changes of blood glucose, plasma lipid and protein levels during fasting were surveyed in sheep and rats.

Blood glucose level seemed to be associated with the change of plasma 11-OHCS level as the results (Exp. I). Since insulin⁴¹⁾, epinephrine^{31,32,41,118)}, glucagon^{31,32,37,41,43,144)}, growth hormone⁴¹⁾, glucocorticoids^{30,41,51,53,99,118,157)}, etc., had been known as the hormones which could regulate blood glucose level, however, it was not certain if the maintained level of blood glucose would be directly related to the increased plasma 11-OHCS.

Glucose was intravenously injected into the fasted sheep and its effects on plasma 11-OHCS, protein and lipid level were examined to elucidate whether adrenal cortex played a direct role in the mechanism for the maintenance of blood glucose level in sheep during fasting or not.

1. Materials and Methods

Experimental animals, sheep (Japanese Corriedale) and rats (Wistar strain), were fed at the Laboratory of Animal Breeding of Miyazaki University. About 16 months old wethers and about 2 months rats were used. The mean body weights with standard deviation were 34.7 ± 4.8 kg in sheep, 308.8 ± 56.1 g in the male rats and 211.3 ± 28.6 g in the female. Each animal was kept individually in a metallic cage for rats and in a pen with sands bedding for sheep. Rats were fed in an environment controlled chamber (Koitoiron), where the temperature was kept at 25 °C and the relative humidity was 60 to 70 %. Water was given *ad libitum* during fasting.

This study consisted of two experiments. Blood samples were taken from each animal (6 heads) at 0, 2, 4, 6, 8, 10, 15, 20 and 30th day after the removal of feeds in sheep, whereas from the different animals

allotted to each particular day during fasting; 0, 2, 4, 6, 8 and 10th day in rats (male, 24 heads; female, 15 heads) (Exp. I). Blood glucose, plasma protein and lipid levels were determined in all samples.

Glucose solution (10 g%, W/V) was intravenously injected two times with the interval of one hour into the sheep fasted for 24 days (Exp. II). The dose for each time was 0.5 mM per kg of the body weight before fasting. It took 5 minutes to inject the dose of glucose. Blood samples were taken at 0, 2, 3, 4, 6 and 28 hours after the first injection of glucose. Blood glucose, plasma 11-OHCS, protein and lipid levels were determined.

Blood glucose, plasma total lipid and protein levels were determined by SOMOGYI method¹³⁹⁾, BRAGDON method¹⁴⁶⁾ and Tsukasa Protein Refractometer, respectively. Densitometric determination of plasma lipid fractions by TLC was carried out according to KANNO *et al*⁶³⁾. Plasma 11-OHCS level was determined by the method described previously (Chapter I).

2 Results

(Experiment I)

Blood glucose, plasma total protein and lipid levels in sheep and rats before the removal of feeds are shown in Table 17.

Table 17 Blood glucose, plasma total protein and lipid level in sheep and rats before the removal of feeds

Animal	Glucose (mg %)	Protein (g %)	Lipids (mg %)
Sheep (6)	47.6 ± 3.9*	5.90 ± 0.33	251.9 ± 15.8
Male(6)	99.7 ± 10.8	6.53 ± 0.35	283.2 ± 63.1
Rat Female(3)	89.2 ± 12.2	7.27 ± 0.25	487.4 ± 18.2

* : Mean ± standard deviation; numbers of animals are given in parentheses

The results in sheep (Figs. 19 and 21) were expressed as relative values, namely, as a percentage for the value before the removal of feeds.

Changes of body weight, blood glucose, plasma total protein and lipid level are shown in Figs. 19 and 20. Body weight gradually decreased during fasting in both sheep and rats as well as in the previous experiment (Chapter VI). Blood glucose level decreased more dramatically for the first 2 days in rats and 4 days in sheep after the removal of feeds than body weight. The mean blood glucose level at the end of each period was significantly lower than the control level ($P < 0.05$). From that time, the blood glucose level was maintained up to the end of fasting, or even elevated at the time.

The change of plasma total protein level in sheep was contrary to that in rats. The protein level increased up to 10 days of fasting in sheep ($P < 0.01$), but began to decrease from 4th day after the removal of feeds in rats and its decrease became significant at 6th day in female ($P < 0.05$).

Plasma total lipid level in sheep began to increase at 2th day of fasting and reached a maximum at 10th day ($P < 0.05$). But the level seemed to decrease gradually thereafter (Fig. 19). Free fatty acids (FFAs) increased remarkably by fasting ($P < 0.01$), while triglyceride somewhat decreased as shown in Table 18. In rats plasma total lipid level decreased dramatically (Fig. 20).

(Experiment II)

The mean plasma 11-OHCS level with standard deviation prior to glucose injection was $4.50 \pm 1.08 \mu\text{g}/100 \text{ ml}$ and it was significantly higher than the level before fasting; $3.00 \pm 0.95 \mu\text{g}/100 \text{ ml}$ ($P < 0.05$).

When glucose was injected into the sheep fasted for 24 days, the blood glucose level changed as shown in Fig. 21. The increase of blood glucose level was accompanied with the significant decline of plasma 11-OHCS level ($P < 0.01$). The blood glucose at 28 hours after the injection declined to the level before the injection, accompanied with the 11-OHCS level recovery.

Also, plasma total protein ($P < 0.01$) and lipid ($P < 0.05$) levels decreased after the glucose injection (Fig. 22). Their decrease seemed to be later than that of plasma 11-OHCS. At that time, only FFAs

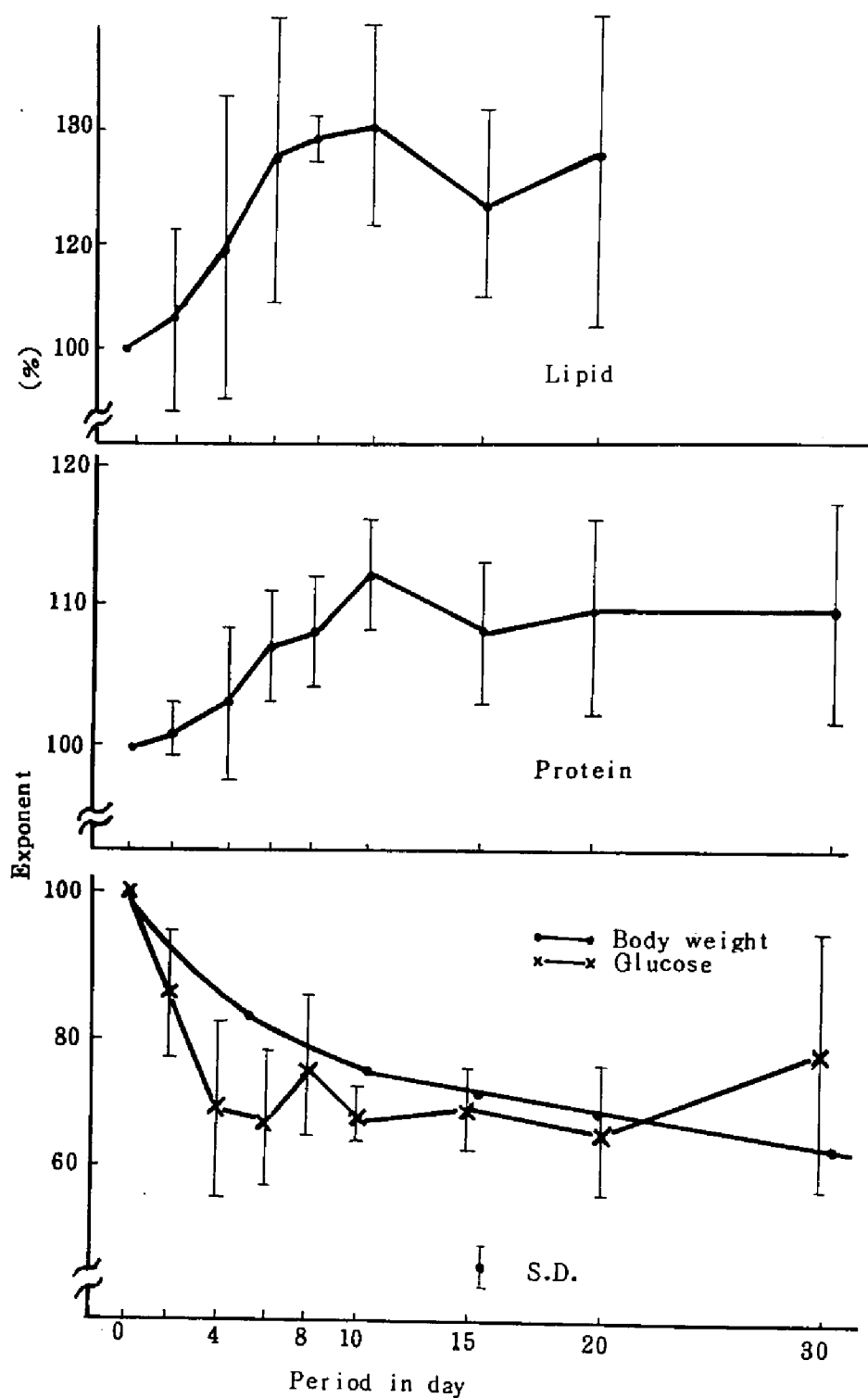


Fig. 19. Changes of body weight, blood glucose, plasma total protein and lipid level during fasting in sheep
Each value is the mean of 6 animals

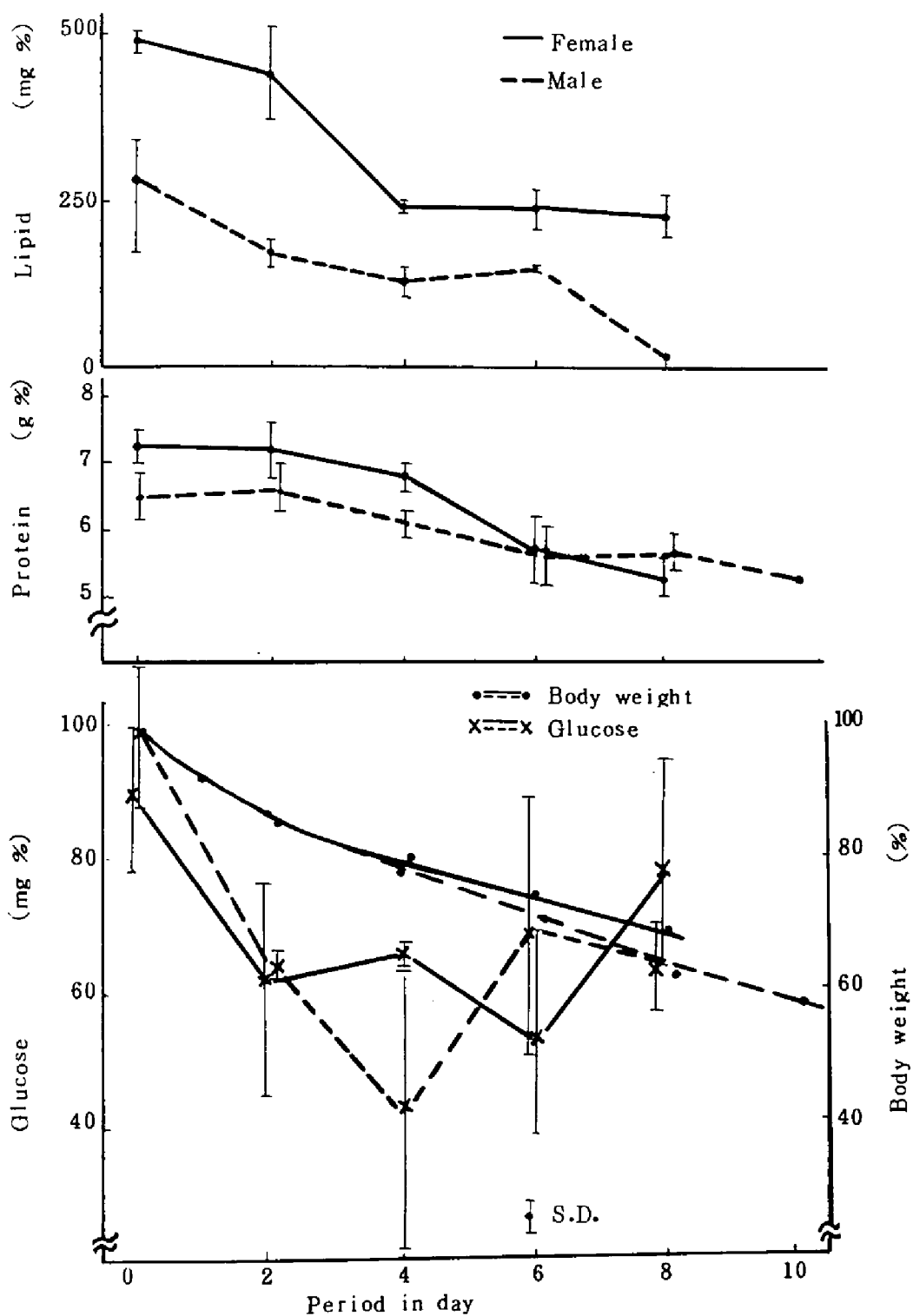


Fig. 20 Changes of body weight, blood glucose, plasma total protein and lipid level during fasting in rats

Each value is the mean of 4 male animals or 3 female

decreased significantly ($P < 0.01$) in the lipids as shown in Table 19.

3 Discussion

A sufficient supply of glucose to the fasted sheep seemed to result in relaxation of hyperadrenocortical activity due to fasting as far as the present experiment was concerned. Moreover, BASSETT⁶⁾ reported that cortisol given to sheep throughout fasting of 10 days increased blood glucose level. These results indicate that adrenal cortex is of particular importance for the maintenance of blood glucose level during fasting in sheep.

Plasma 11-OHCS level did not increase significantly during fasting in rats (Chapter VI). However, blood glucose level was maintained up to 8 days except for the decrease of the first 2 days after the removal of feeds as well as in sheep. Some hormones secreted from other than

Table 18. Effect of fasting on plasma lipid fraction level in sheep

Day of fasting	Cholesterol ester	Tri- glyceride	Free fatty acids	Cholesterol	Phospho- lipid
0	57±21	47±21	10±7 ^a	46±7	92±7
2	39±11	35±7	71±30 ^b	50±14	75±16
4	46±14	36±19	83±32 ^b	50±16	80±13
6	45±16	39±23	101±30 ^b	68±13	74±22
8	54±20	28±16	108±17 ^b	71±9	73±17
10	58±15	30±8	104±15 ^b	64±11	84±22
15	55±7	21±6	84±17 ^b	63±9	83±16
20	42±5	29±12	107±21 ^b	63±13	81±24

(Unit mg %)

a and b : Differences between means in a column with the different letter are significant ($P < 0.01$)

Each value is the mean with S.D. of 4 animals

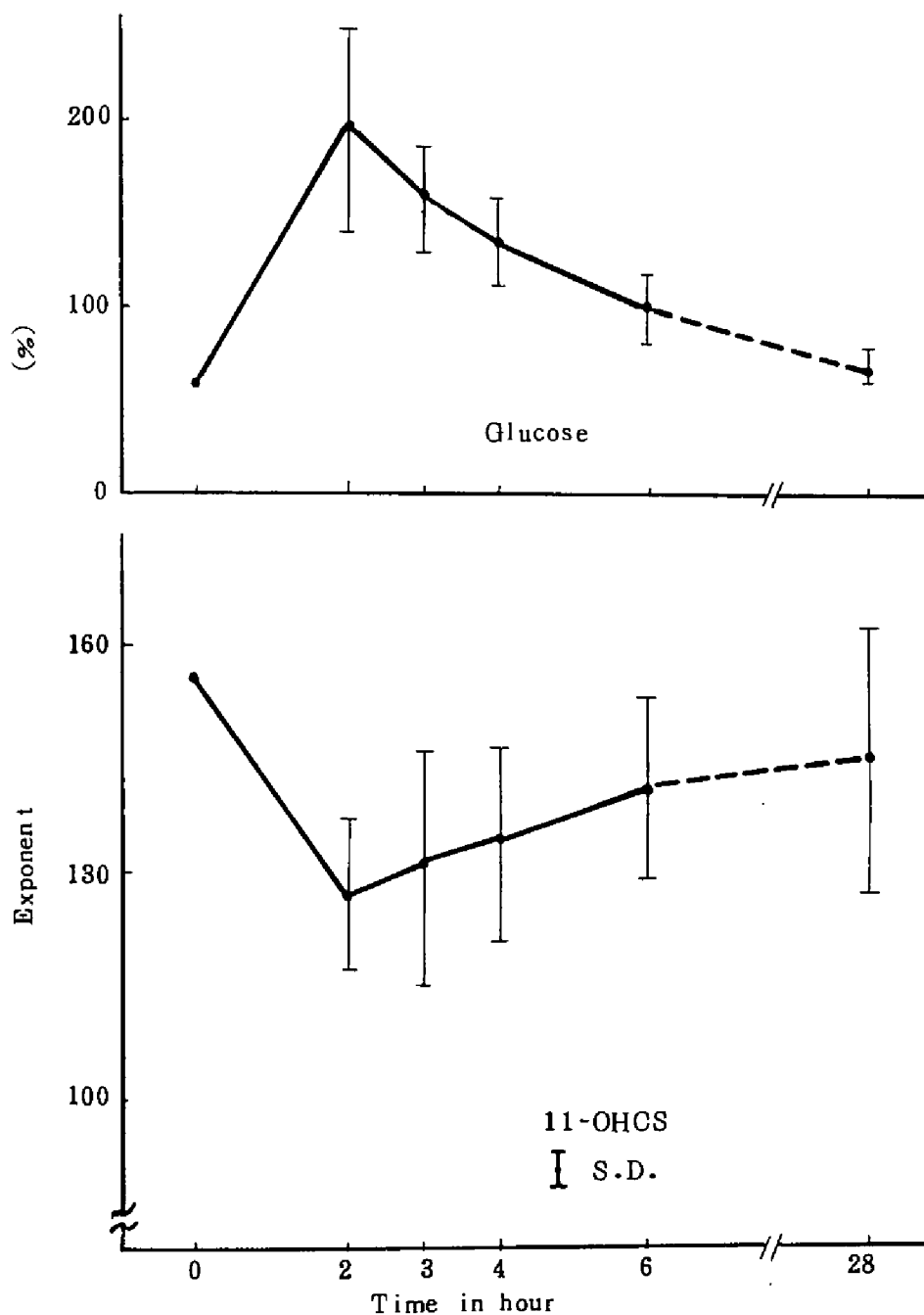


Fig. 21. Changes of blood glucose and plasma 11-OHCS level after glucose injection in the fasted sheep

Each value is the mean of 5 animals; the initial value before the removal of feeds is 100

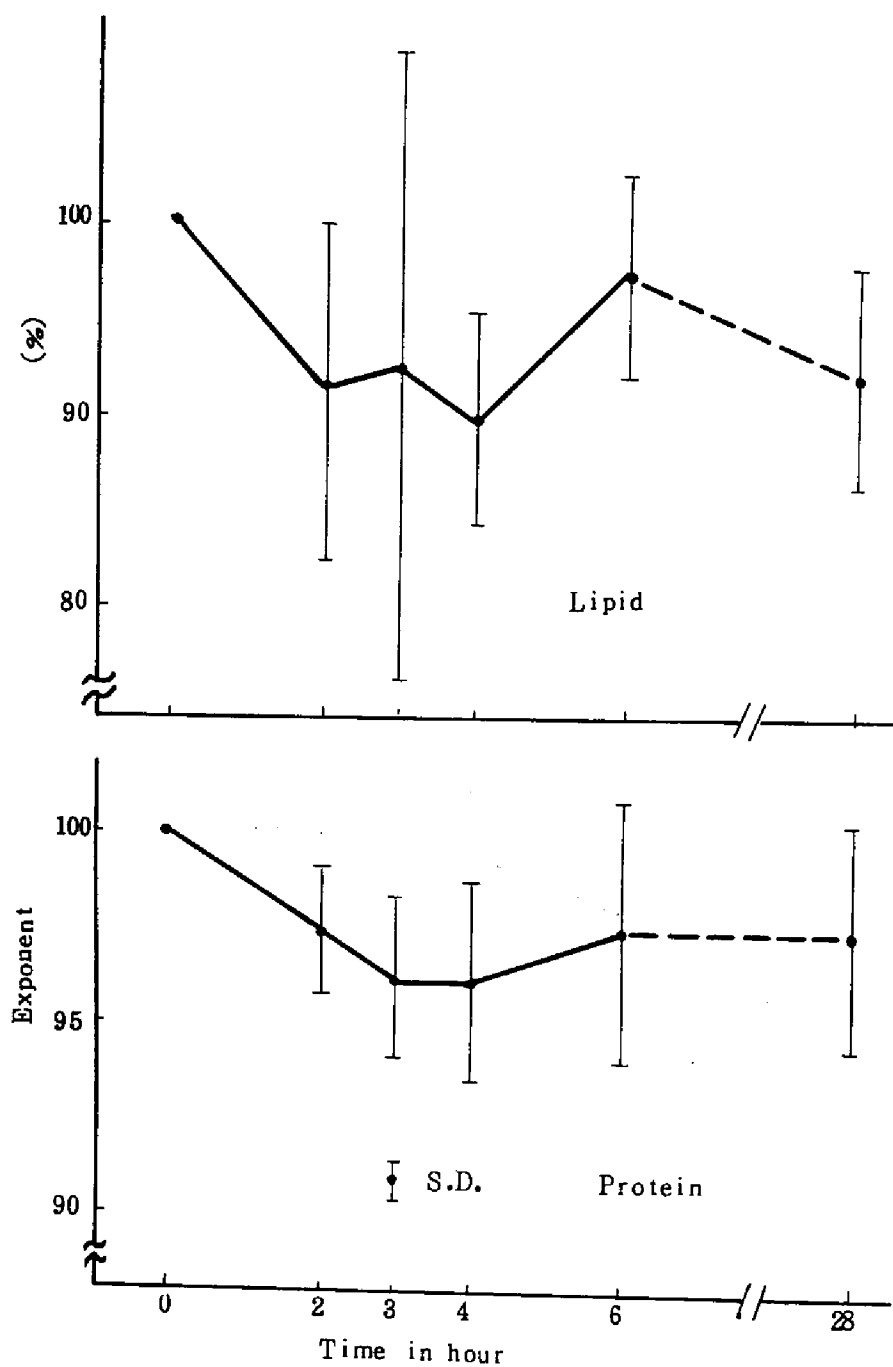


Fig. 22. Changes of plasma total protein and lipid level after glucose injection in the fasted sheep

Each value is the mean of 5 animals

Table 19. Effect of glucose injection on plasma lipid fraction level in the fasted sheep

Time after injection	Cholesterol ester	Tri-glyceride	Free fatty acids	Cholesterol	Phospho-lipid
0	65 ± 22	39 ± 16	87 ± 27 ^a	64 ± 15	60 ± 14
3	73 ± 34	47 ± 22	51 ± 8 ^c	65 ± 15	59 ± 11
6	68 ± 19	47 ± 16	74 ± 28 ^{ab}	69 ± 23	59 ± 10
28	64 ± 13	43 ± 10	72 ± 16 ^b	59 ± 17	57 ± 19

(Unit mg%)

a, b and *c* : Differences between means in a column with the different letter are significant ($P < 0.01$)

Each value is the mean with S.D. of 5 animals

adrenal cortex may act for the maintenance of blood glucose level in rats during fasting, though the participations of glucocorticoids in the maintenance cannot be absolutely ruled out. For example, epinephrine^{31, 32, 118}), glucagon^{31, 32, 37, 43, 144}), etc., also have the gluconeogenetic action. Moreover, YALOW *et al.*¹⁶⁷) suggested that both sensitivity and secretion rate of insulin in rats were reduced by fasting. This insensitivity of insulin seems to be due to alterations in secretion of adrenal medullary hormones according to FELDMAN³⁵). These hormones may either independently or co-operatively act to maintain blood glucose level in rats.

It is interesting that FFAs increased rapidly after the removal of feeds, but they were reduced following the decrease of plasma 11-OHOS level by the glucose injection. It has been shown the similar results in sheep^{6, 61}) and in dogs¹⁴²). TRENKLE¹⁵³) reported that an intravenous injection of glucose into fasting sheep decreased the level of plasma FFAs within 1 hour.

Non-esterified fatty acid level in plasma increases with the body need for energy and the utilization of the fatty acids is proportional to its level in sheep¹²²). The increase of plasma FFA level in sheep seems to represent active fatty acid mobilization. The mobilized fatty

acids may be utilized in the tissue other than brain, nervous system, etc., as a source of energy^{48,142)}, and may aid in saving of glucose. In facts BASSETT *et al.*⁸⁾ reported that fasting diminished the glucose utilization rate in sheep. It is suggested from the present results that plasma 11-OHCS may also indirectly contribute to the maintenance of blood glucose level through fat mobilization in sheep.

Blood glucose was not depressed under a certain level in spite of the prolonged fasting in both sheep and rats. However, the underlying mechanism for the maintenance of blood glucose level could be different between the two animal species tested. Moreover, it is suggested that glucocorticoids would play more important roles in the mechanism in sheep than in rats.

Chapter VIII. Effect of ACTH on plasma 11-OHCS level and F/B ratio

Since normal plasma 11-OHCS level is very low in ruminants, the level of 11-OHCS should be augmented to get more precise information of the adrenocortical function of them. In the present study, the response of plasma 11-OHCS to exogenous ACTH was investigated in order to examine responsibility of adrenal cortex itself in the hypothalamic-hypophyseal-adrenocortical system. Many references about this type of research could be easily found in cattle^{17,120,161}), sheep^{7,124}), humans¹⁰⁹), rabbits⁶⁵), chickens⁹⁵) and rats^{47,93}). However, the data compiled from these reports were equivocal, and were not sufficient enough to depict species specificity, if present.

In the present experiments plasma 11-OHCS level and F/B ratio were determined to clarify quantitative and qualitative differences in the responses of plasma 11-OHCS to ACTH.

1. Materials and Methods

Young fattening steers (Japanese Black), adult wethers (Japanese Corriedale), adult male rabbits (Japanese native) and adult rats (Wistar strain) were used. Body weights and numbers of the animals are shown in Table 20.

This investigation consisted of 4 experiments. Changes of plasma 11-OHCS level after ACTH injection were investigated in Exp. I. The dose of ACTH to each species was adopted as shown in Table 20, based on the results by some researchers^{5,124,148}). After an injection of ACTH, blood samples were taken with appropriate time intervals from each cattle and sheep, but from different individuals allotted to the scheduled time intervals in rabbits and rats. Physiological saline (0.9 %) had been consecutively injected 3 to 5 days in rabbits and 10 days in rats to train the animals not influencing by the treatment (BARRETT *et al.*⁵). The animals were also similarly trained in Exps. III and IV.

Exp. II was undertaken to know the dose-response of ACTH and plasma 11-OHCS levels in sheep by giving graded doses of ACTH (0, 5, 10 and

Table 20. Body weight and metabolic body weight of animals, and dose of ACTH

Animal	Number	Body Weight (kg)	Metabolic Body Weight (kg)	Dose of ACTH (I.U./head)	
				Dose shown in the past literatures	Adjusted Doses ^d
Cattle	6	380	86	40 ^{a)}	34
Sheep	8	43	17	20 ^{b)}	6.5
Rabbit	7	2.7	21	4	0.8
Rat	5	0.31	0.42	1 ^{c)}	0.17

a), b) and c): These values were quoted from the reports by TAKEUCHI¹⁴⁸⁾, SABA¹²⁴⁾, BARRETT *et al.*⁵⁾, respectively, and 4 I.U./head in rabbits was tested beforehand by the author. These doses of ACTH sufficed to increase plasma 11-OHCS level of each animal

d): 0.40 I.U. of ACTH per kg of metabolic body weight was used as a common standard unit for every animal in order to calculate this adjusted doses

20 I.U. per head).

Exps. III and IV were conducted, based on the results of these two preliminary experiments. In Exp. III, 0.40 I.U. per kg of metabolic body weight was selected as a standard dose of ACTH for all animals. The adjusted doses are described in Table 20. Blood samples were taken from cattle, sheep, rabbits and rats at 2, 2, 1 and one-half hour(s), respectively, after the injection of either ACTH or saline as shown in Table 21.

In Exp. IV, doses of ACTH increased plasma 11-OHCS levels were injected into cattle, sheep, rabbits and rats. And the effect on F/B ratio in plasma was investigated.

ACTH preparation used in the present study was ACTH 'Daiichi'*. Either ACTH or saline was injected intraperitoneally. Plasma samples

* : ACTH 'Daiichi' is a commercial preparation of ACTH by Daiichi Seiyaku Co., Ltd, in Japan.

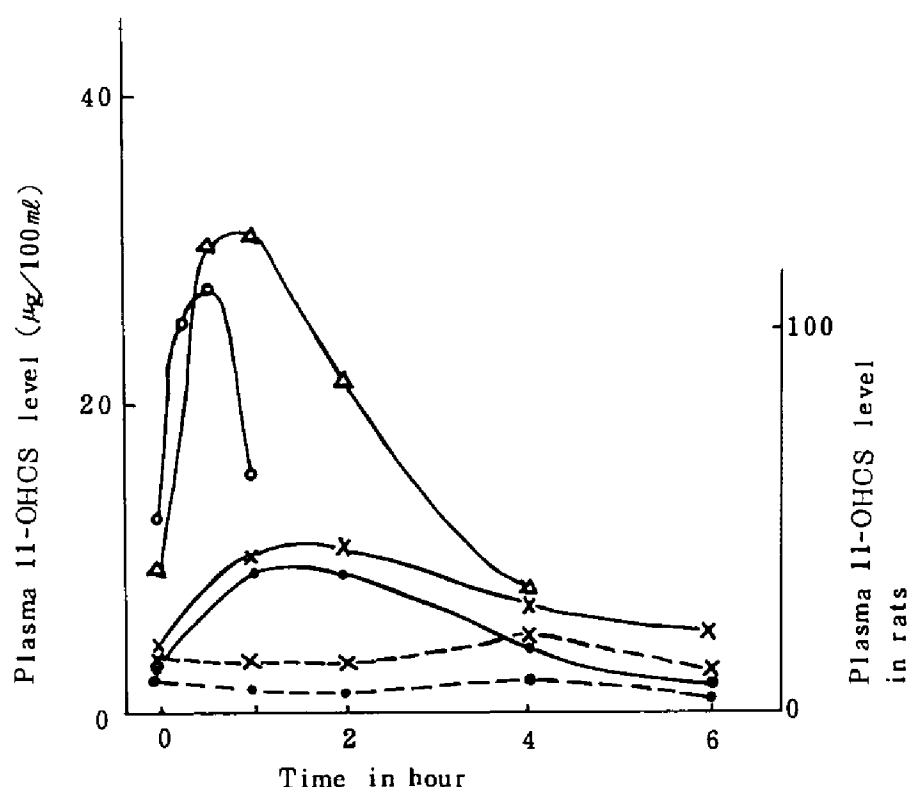


Fig. 23 Changes of plasma 11-OHCS levels in cattle, sheep, rabbits and rats by the time after an intraperitoneal injection of either ACTH or saline (Exp. I)

Solid line (—) is for injection of ACTH*; broken one (---) is for injection of saline. And solid dots (●) represent the mean level of cattle; crosses (×) represent the mean level of sheep; open circles (○) represent the mean level of rats; triangles (△) represent the mean level of rabbits

*: The dose of ACTH per head was 40 I.U. in cattle, 20 I.U. in sheep, 4 I.U. in rabbits or 1 I.U. in rats

were collected and stored in the same way as previously described (Chapter I). Plasma 11-OHCS levels and F/B ratios were determined by the methods presented previously (Chapter I).

3. Results

The results of Exp. I are shown in Fig. 23. The saline injection had no effect on plasma 11-OHCS levels for six hours in cattle and sheep. An injection of ACTH increased evidently plasma 11-OHCS levels in all animals.

In Exp. II, the levels of plasma 11-OHCS in sheep reached the maximum value at two hours after ACTH injection even though the dose of ACTH was different (Fig. 24).

The results of Exp. III are shown in Table 21. High basal plasma 11-OHCS levels in rats were not modified by the ACTH injection, while the levels in rabbits, cattle and sheep were significantly elevated.

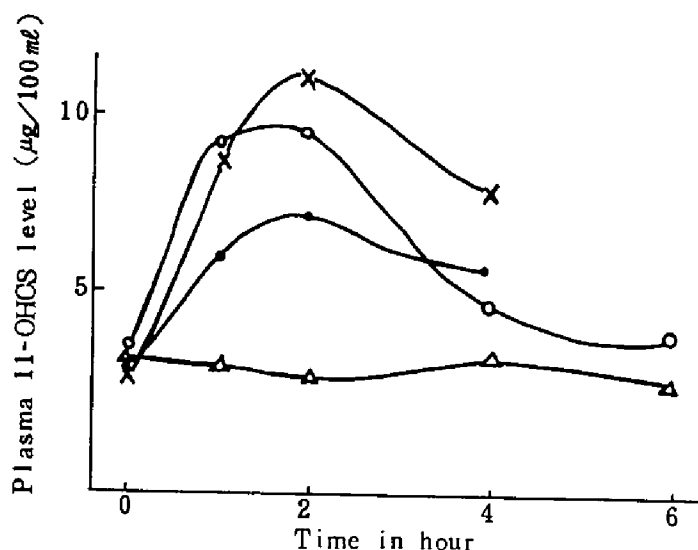


Fig. 24 Changes of plasma 11-OHCS levels in sheep by the time after an intraperitoneal injection of various doses of ACTH (Exp. II)

Each mark shows mean plasma 11-OHCS level after injection of the corresponding dose of ACTH; open circles (○) 20 I.U./head, crosses (×) 10 I.U./head, solid dots (●) 5 I.U./head and triangles (△) saline

Table 21. Plasma 11-OHCS levels in cattle, sheep, rabbits and rats at the time of peak after an intraperitoneal injection of either ACTH or saline (Exp. III)

Animal Number	Plasma 11-OHCS level $\pm$ S.D.		An index of response intensity ($y/x \pm$ S.D.)	Elapsed time (hour) from injection to blood sampling*
	Saline ($x, \mu\text{g}/100\text{ml}$)	ACTH* ($y, \mu\text{g}/100\text{ml}$)		
Cattle 6	174 $\pm$ 049	953 $\pm$ 154	5.82 $\pm$ 1.63 ^a	2
Sheep 8	298 $\pm$ 097	838 $\pm$ 308	3.26 $\pm$ 1.98 ^b	2
Rabbit 7	863 $\pm$ 42	2686 $\pm$ 32	3.81 $\pm$ 1.77 ^b	1
Rat 5	3064 $\pm$ 132	2984 $\pm$ 43	1.21 $\pm$ 0.69 ^c	1/2

a, b and c: Differences between means in a column with the different letter are significant ($P < 0.05$)

S.D.: Standard deviation

* : The mean dose of ACTH per head was 34 I.U. in cattle, 6.5 I.U. in sheep, 0.8 I.U. in rabbits or 0.17 I.U. in rats

** : This is the time required to reach the maximum plasma 11-OHCS level in Experiment I

The percentages of plasma 11-OHCS after the injection to the initial levels were 582 in cattle, 326 in sheep, 381 in rabbits and 121 in rats. And the differences among animal species were statistically significant ($P < 0.05$), except for the difference between sheep and rabbits.

F/B ratios in plasma of sheep at the peak of 11-OHCS level after the ACTH injection and those in the untreated are shown in Table 22. The latter value were quoted from the previous data (Chapter III). There was a tendency that the F/B ratios in cattle and sheep somewhat increased after ACTH injection, while those in rabbits and rats somewhat decreased.

4. Discussion

The time required to reach the maximum plasma 11-OHCS level after ACTH injection was two hours in cattle and sheep, one hour in rabbits and thirty minutes in rats. An argument, whether the chronological

Table 22. Acute effect of an intraperitoneal injection of ACTH on the ratio of cortisol to corticosterone (F/B ratio) in plasma of cattle, sheep, rabbits and rats (Exp. IV)

Animal	F/B ratio	
	Untreated*	ACTH injection
Cattle	3.6	4.2
Sheep	3.8	9.6
Rabbit	1.9	0.3
Rat	0.4	0.2

* : These values were cited from the previous chapter (III)

pattern of this response is characteristic of each species or not, has not been settled. There were reports that it took one¹⁶¹⁾ and two hours¹²⁰⁾ in cattle to reach the maximum of plasma 11-OHCS level. The time was six hours and forty minutes in sheep¹²⁴⁾, and one hour⁹³⁾ or a quarter of an hour⁴⁷⁾ in rats. In the present experiment, the time required in sheep was constantly two hours regardless of the various doses of ACTH. Similar results have been reported in rats by GUILLEMIN *et al.*⁴⁷⁾, in which the time was always a quarter of an hour at various dose levels of ACTH given. These results were consonant with an idea that the reaction time for ACTH might be fixed in certain species, at least, in rats and sheep.

In Exp. III, a common standard dose was set to carry out reasonable comparison of the sensitivity of plasma 11-OHCS to exogenous ACTH among animal species. Plasma 11-OHCS levels in each species after the saline injection were found in a range of resting condition in all species (Chapter III), indicating that the elevated plasma 11-OHCS after the ACTH injection was entirely attributable to exogenous ACTH. Based on these findings, an index as presented in Table 21 was proposed to help comparative study of the mode of ACTH dependent responses among species. It was reported by GUILLEMIN *et al.*⁴⁷⁾ that the index values were 114 and

136 in rats received 0.05 mU or 0.1 mU of ACTH, respectively. The results were in agreement with those of the present results in rats. It is suggested that the response of adrenal cortex to ACTH is different among animal species with the order of cattle > sheep and rabbits > rats.

The predominant corticoid in each animal seemed to be preferably increased by the ACTH injection in this study. KASS *et al.*⁶⁵⁾ reported that a single administration of ACTH did not alter significantly F/B ratio, though the administrations for a prolonged period increased the ratio in rabbits. According to BUSH¹⁸⁾, F/B ratio was not affected by ACTH treatment in some animals. Exceptionally, the ratio was reduced from 5.0 to 2.35 by ACTH in the cat. FRASER³⁶⁾ reported that ACTH reduced the ratio in humans. On the contrary, F/B ratio was remarkably elevated by ACTH injection in cattle¹⁶¹⁾. It is suggested from these results that there may exist a species difference in the response of F/B ratio to ACTH.

Chapter IX. Effect of ACTH, cortisol and corticosterone injection on blood eosinophils count

THORN *et al.*¹⁵¹⁾ reported that injection of ACTH reduced the peripheral blood eosinophils count in humans. After that, this fact was confirmed in some other animals^{29,89,102)}. However, the responses seem to be species specific, *i.e.*, ACTH induced eosinopenia in cattle⁸⁹⁾, goats¹⁰²⁾, humans^{54,62,151)} and guinea pigs²⁹⁾, but eosinophilia in chickens⁹⁷⁾.

The effect of ACTH was observed in the following animals; cattle, sheep, rabbits and rats. Moreover, the cause of this species difference was investigated from the viewpoint of differential action of cortisol and corticosterone.

I. Materials and Methods

Animals used in this investigation were the same as previously described (Chapter VIII).

The present study consisted of 4 experiments. ACTH was injected into cattle and rats at the doses of 40 I.U. and 1 I.U. per head, respectively. In sheep, three different doses of ACTH (5, 10 and 20 I.U. per head) were administered. Blood samples were taken at 0, 2, 4, 6 and 8 hours from each cattle, and at 0, 2, 4 and 6 hours from each sheep after an injection of either saline or ACTH. And it was made at 0, 2, 4 and 6 hours from the different rats allotted to the scheduled time intervals after the injection (Exp. I).

In Exp. II, eosinophils were counted at 0 and 8 hours in cattle, at 0 and 6 hours in sheep, at 5 hours in rabbits and at 0, 2, 4 and 6 hours in rats after the injection of either saline or ACTH. The doses were the same as Exp. I. The saline had been consecutively injected 3 to 5 days in rabbits and 10 days in rats to train the animals not influencing by the treatment. The training for rats was also practiced in Exps. I, III and IV.

In Exp. III, six heads of sheep were allotted to three groups received saline, cortisol or corticosterone. All these treatments were given in order each group every week. The fifteen rats were divided into 3 groups.

Table 23. The dose of an injected hormone and the base for calculation

Item	Sheep	Rat
A ratio of total blood volume to body weight	1/12	1/15
Plasma 11-OHCS level* (μg/100ml)	4	10
Dose (mg/kg B.W.)	2.0	4.0

*: Plasma 11-OHCS levels in sheep and rats at the same conditions as the animals used in this experiment were 3.68 and 9.12 μg/100ml, respectively

The saline, cortisol and corticosterone were injected for each group; 3, 6 and 6 rats, respectively. After one week, a treatment differed from that at the first time was given each group. As a control, eosinophils were counted in other six rats before the treatment. The doses were 600 times amount of the total 11-OHCS presumed to be existed in the whole blood; 2.0 mg and 4.0 mg per kg of body weight in sheep and rats, respectively. These doses were calculated basing on the standards shown in Table 23. Blood samples were taken at 0, 2, 4 and 6 hours from each sheep and at 1, 2 and 4 hours from the different rats allotted to each time after the injection of saline, cortisol or corticosterone.

In Exp. V, three ewes received saline, 25 mg and 100 mg of cortisol per individual, respectively. Either cortisol or corticosterone of 2.0 mg per kg of body weight was injected into male rats. Blood samples were collected in the same way as described in Exp. III.

The rats in Exps. III and IV were fed in an environment controlled chamber (Koitozon), where the temperature was set to 25°C and the relative humidity was 50 to 60 %.

ACTH preparation used in the present study was ACTH 'Daiichi'*. The commercial 'Hydrocorton'** was utilized as cortisol preparation.

*; ACTH 'Daiichi' is a commercial preparation of ACTH by Daiichi Seiyaku Co., Ltd. in Japan.

** ; 'Hydrocorton' is a commercial preparation of cortisol by Japanese Merk & Co., Inc..

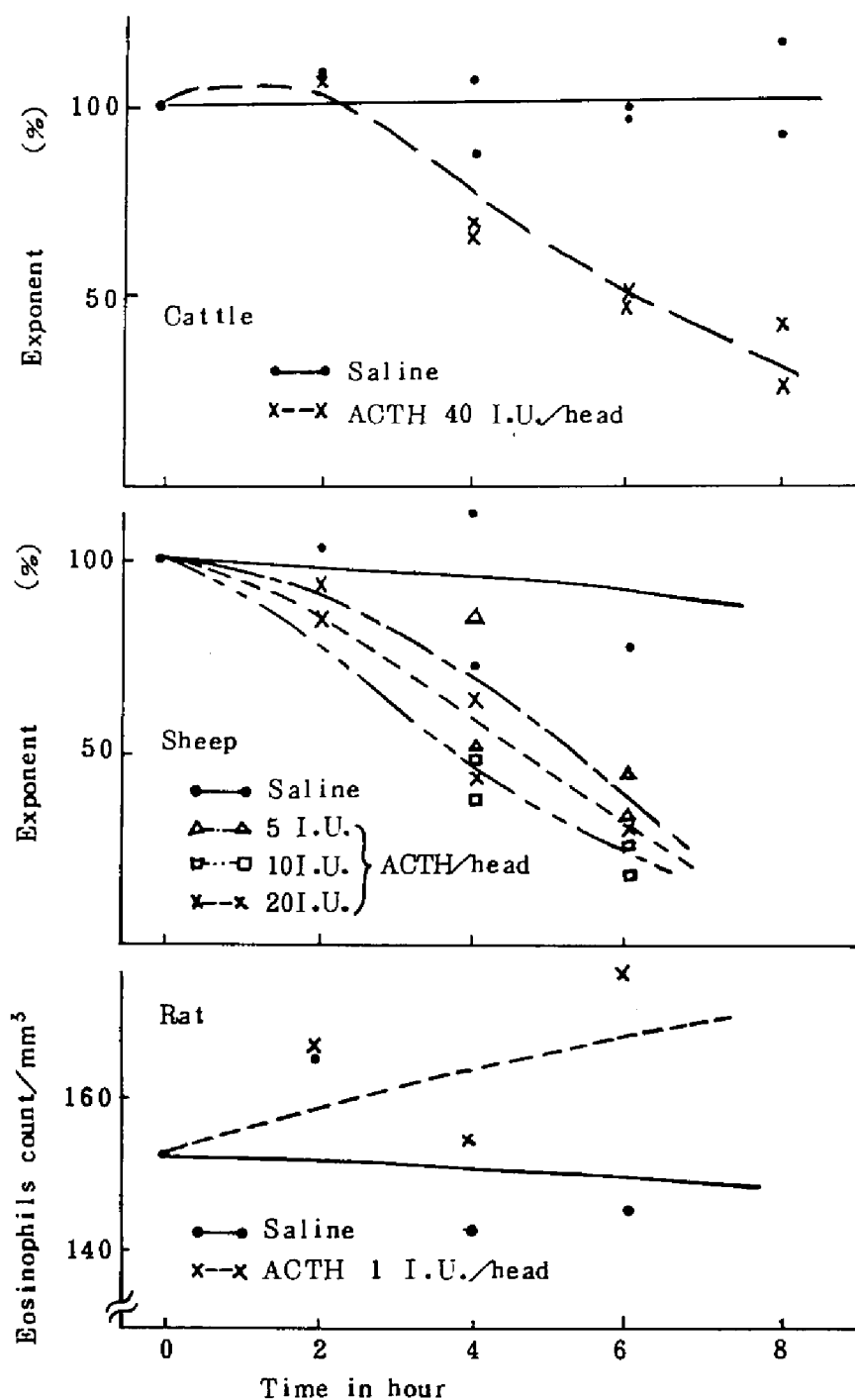


Fig. 25. Changes of the peripheral blood eosinophils counts in cattle, sheep and rats after an intraperitoneal injection of either ACTH or saline (Exp. I)

Corticosterone was suspended in the saline contained one percent of carboxymethylcellulose. Either a drug or saline was injected intraperitoneally. All these experiments usually started at 9:00 to 10:00 A.M..

Blood was collected from jugular vein in cattle and sheep, from the tail vein in rats and from the ear vein in rabbits. Eosinophils were counted by HINKLEMAN's method¹⁰²⁾, using a hemocytometer of Fucks-Rosenthal type.

2. Results and Discussion

The results of Exp. I are shown in Fig. 25. The eosinophils count was not modified remarkably by the saline injection in all animals tested. The ACTH injection decreased eosinophils count in cattle and sheep as the time proceeded, while it increased somewhat in rats.

The effects of either saline or ACTH injection in Exp. II are shown in Table 24. The eosinophils count was not modified by the saline injection. The counts decreased significantly in both cattle and sheep injected with ACTH ($P < 0.01$), but the counts increased significantly in rabbits ($P < 0.01$) and were not changed in rats.

The species difference regarding the response of eosinophils to ACTH was supposed to be existed, based on the results by several researchers (29,54,62,89,97,102,151). It suggests that animal species having cortisol predominantly in plasma might show severe eosinopenia to ACTH, while the species having corticosterone might not. The results of Exp. II gave further support to this suggestion.

Since the F/B ratio in plasma seemed to be unchanged by the ACTH injection as described previously (Chapter VII), the differential actions of cortisol and corticosterone rather than the qualitative differences in glucocorticoids among animals could be related to the eosinophil pattern specific to the species.

The effects of cortisol and corticosterone on eosinophils were investigated in both sheep and rats (Exp. III). Control eosinophils counts per mm³ (Mean $\pm$ S.D.) were 125 ± 17 , 92 ± 20 and 72 ± 32 in three groups of sheep, and 150 ± 39 in rats. These values were similar to the results in Exp. II and also agreed with the results by BLENKINSOPP and BLENKINSOPP¹³⁾.

Table 24. Effect of an intraperitoneal injection of either ACTH or saline on the peripheral blood eosinophils count in cattle, sheep, rabbits and rats (Exp. II)

Animal	Eosinophils Counts (per mm ³)			
	Pre-Injection	Post-Injection		
		Saline	ACTH	
Cattle	312±124 ^a (8)	381± 85 ^a (4)	85± 58 ^b (5)	
Sheep	102± 93 ^a (10)	123± 38 ^a (5)	15± 8 ^b (5)	
Rabbit		7,180±1,879 ^a (5)	10,578±1,991 ^b (4)	
Rat	152± 73 ^a (6)	152± 28 ^a (5)	163± 60 ^a (13)	

^a, ^b: Differences between the means having the different letter in the same species are significant (P<0.01)

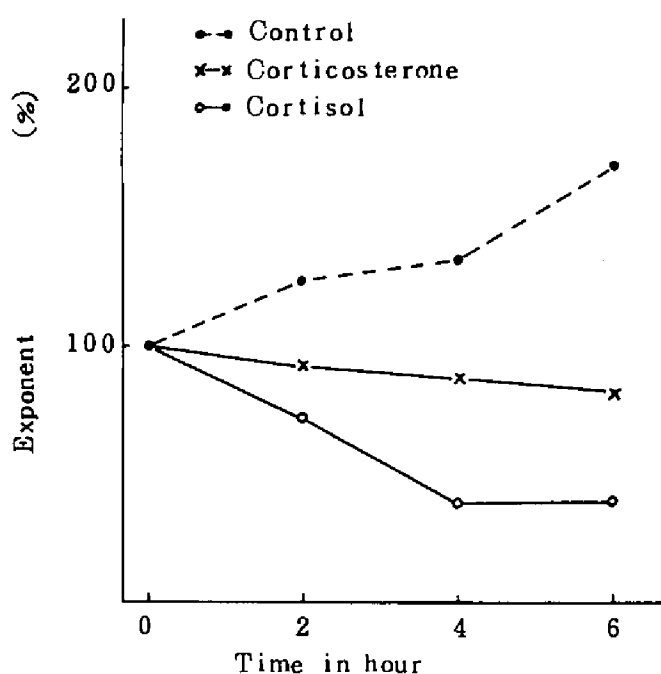


Fig. 26. Effect of an intraperitoneal injection of saline, cortisol or corticosterone on blood eosinophils counts in sheep (Exp. III)

Each value is the mean of six animals

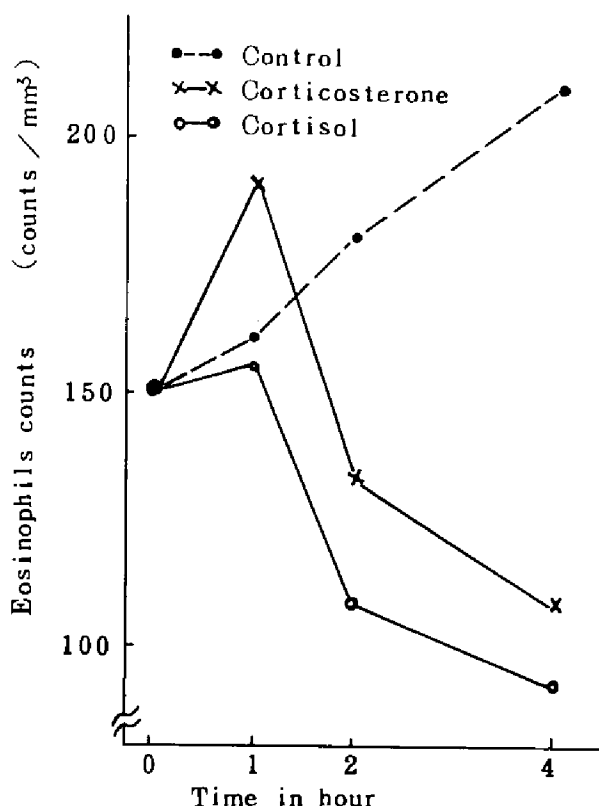


Fig. 27 Effect of an intraperitoneal injection of saline, corticosterone (4.0mg/kg. B.W.) on blood eosinophils counts in rats (Exp. III)

Each value is the mean of 2 to 6 animals

After the injection of saline, eosinophils count increased and reached 165 % of the initial value in sheep and 140 % in rats. On the other hand, the injection of cortisol induced marked eosinopenia in both animals as shown in Figs. 26 and 27. Eosinophils counts were about 40 % of the initial value at 4 to 6 hours in sheep after the treatment, and about 65 % at 2 to 4 hours in rats. The degree of decrease was severer in sheep than in rats. After the corticosterone injection, eosinophils counts decreased and reached 73 % of the initial value at 4 hours in rats, but were not changed in sheep. It has been known that glucocorticoids also induce eosinopenia in humans⁵⁸⁾, guinea pigs²⁹⁾, rats¹³⁾ and goats¹⁰⁰⁾.

It is suggested that both cortisol and corticosterone could reduce

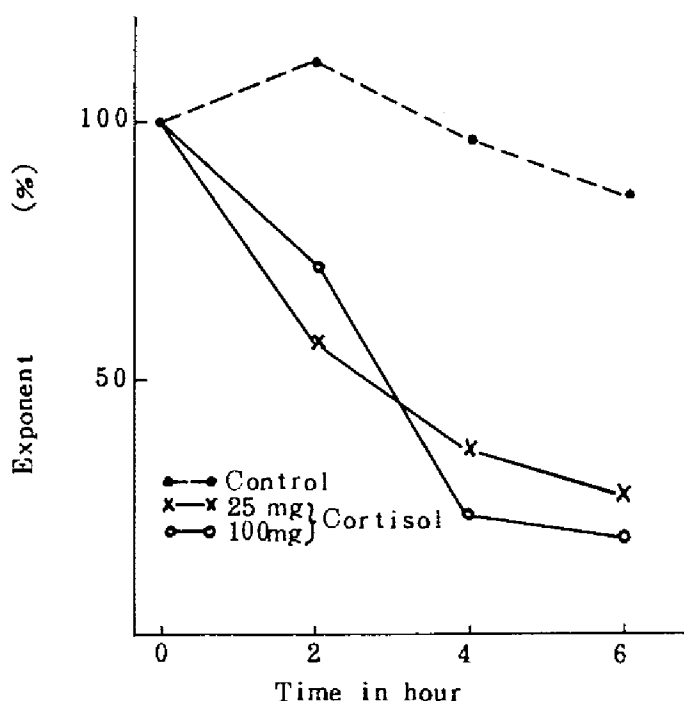


Fig. 28. Effect of an intraperitoneal injection of either saline or cortisol on blood eosinophils counts in sheep (Exp. IV)

eosinophils in sheep and rats, and that the reducing activity of cortisol would be higher than that of corticosterone.

The injection of 25 mg or 100 mg of cortisol to individual sheep decreased eosinophils count (Fig. 28). It was similar to the results of Exp. III, used about 70 mg of cortisol per head. These results suggested that 25 mg of cortisol per head, *i.e.*, about 0.7 mg per kg of body weight, would result in the maximum decrease of eosinophils. But 2.0 mg of cortisol or corticosterone per kg of body weight did not decrease the eosinophils in rats as shown in Fig. 29, indicating that more than 4.0 mg of the hormones would be required to induce eosinopenia.

Eosinopenia was also induced in sheep, even if the dose of cortisol was reduced to about one third of the previous case. Therefore, the eosinophils in sheep would be more receptive to glucocorticoids than those in rats.

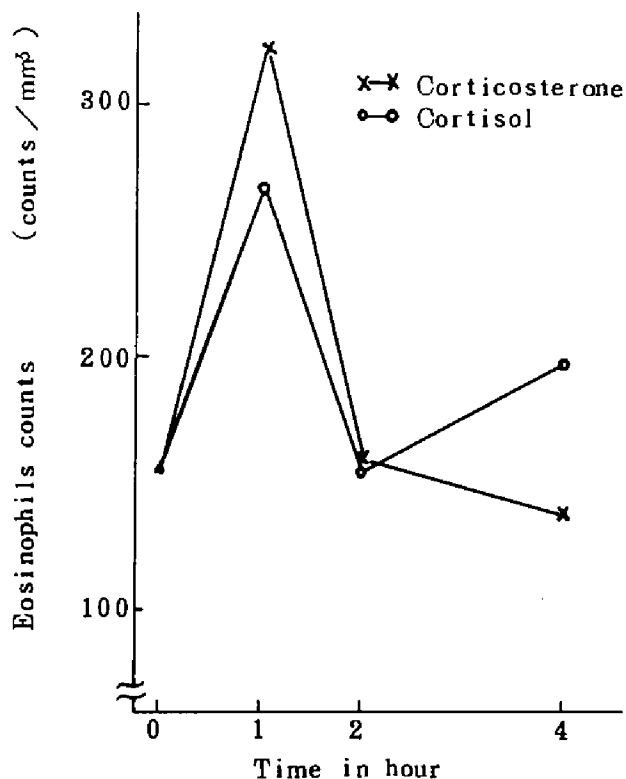


Fig. 29. Effect of an intraperitoneal injection of either cortisol or corticosterone (2.0 mg/kg. B.W.) on blood eosinophils counts in rats (Exp. IV)

Each value is the mean of two animals

It can be concluded that the species specificity in response of eosinophils to the ACTH injection results from a difference in activity between cortisol and corticosterone, and in receptivity between sheep and rats, regarding the effects of the hormones on the eosinophils.

Chapter X. Effect of cortisol and corticosterone on blood glucose, protein and lipid level

The results of previous experiments suggested that the adrenocortical role related to nutrition was more important than the protective action from stresses in ruminants, compared with monogastric animals. As indicated in Chapter IX, there might exist some differences in activity of cortisol and corticosterone, or in receptivity of animal body to the hormones.

The changes of blood glucose, protein and lipid level following either cortisol or corticosterone injection were further investigated in sheep and rats.

1. Materials and Methods

Animals used in this experiment were about one year old wethers and 2 months male rats. Blood samples were collected at the same time in Exp. III of Chapter IX.

Bleeding technique and recipe for blood plasma were described previously (Chapter I). Blood glucose and plasma total protein levels were determined immediately after the bleeding by SOMOGYI method¹³⁹⁾ and Tsukasa Protein Refractometer, respectively. Plasma total lipid level was determined by BRAGDON method¹⁴⁶⁾.

Table 25. Blood glucose, plasma total protein and lipid level in sheep and rats before treatment

Animal	Glucose (mg %)	Protein (g %)	Lipids (mg %)
Sheep(6)	46.3 ± 3.5 *	5.53 ± 0.56	207.3 ± 32.6
Rat(4)	85.7 ± 4.3	6.70 ± 0.18	275.4 ± 28.6

*: Mean ± standard deviation; numbers of animals are given in parentheses

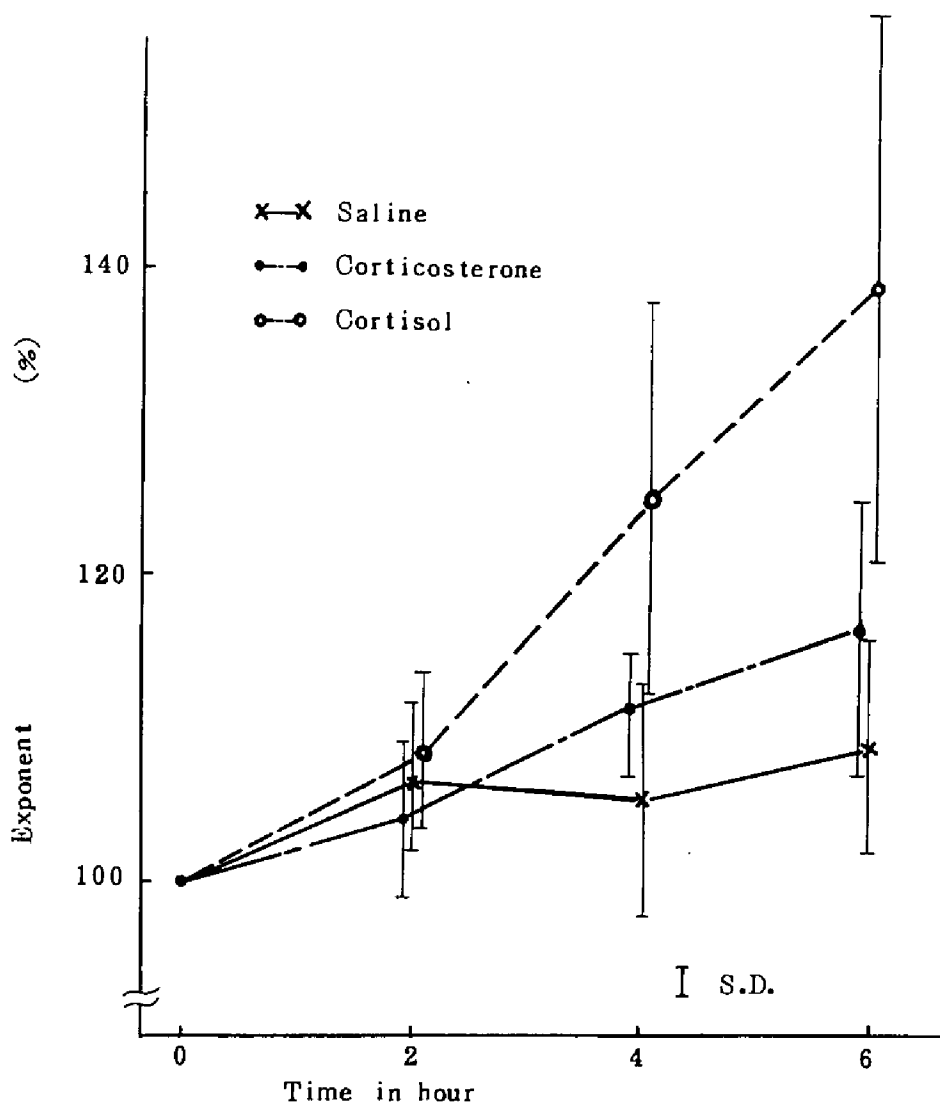


Fig. 30 Influence of an intraperitoneal injection of saline, corticosterone or cortisol on blood glucose level in sheep

Each value is the mean of six animals

2. Results and Discussion

Blood glucose, plasma total protein and lipid levels in sheep and rats before the treatment are shown in Table 25.

The effects of each treatment in sheep (Figs. 30, 31 and 32) are expressed as relative values, namely, as a percentage for the initial value before the treatment. All treatments increased blood glucose level in sheep. Cortisol was the most effective, and corticosterone was intermediate between cortisol and saline. Significant differences were observed between the cortisol- and saline-treated group ($P < 0.01$), and between the cortisol- and corticosterone-treated group ($P < 0.05$), up to

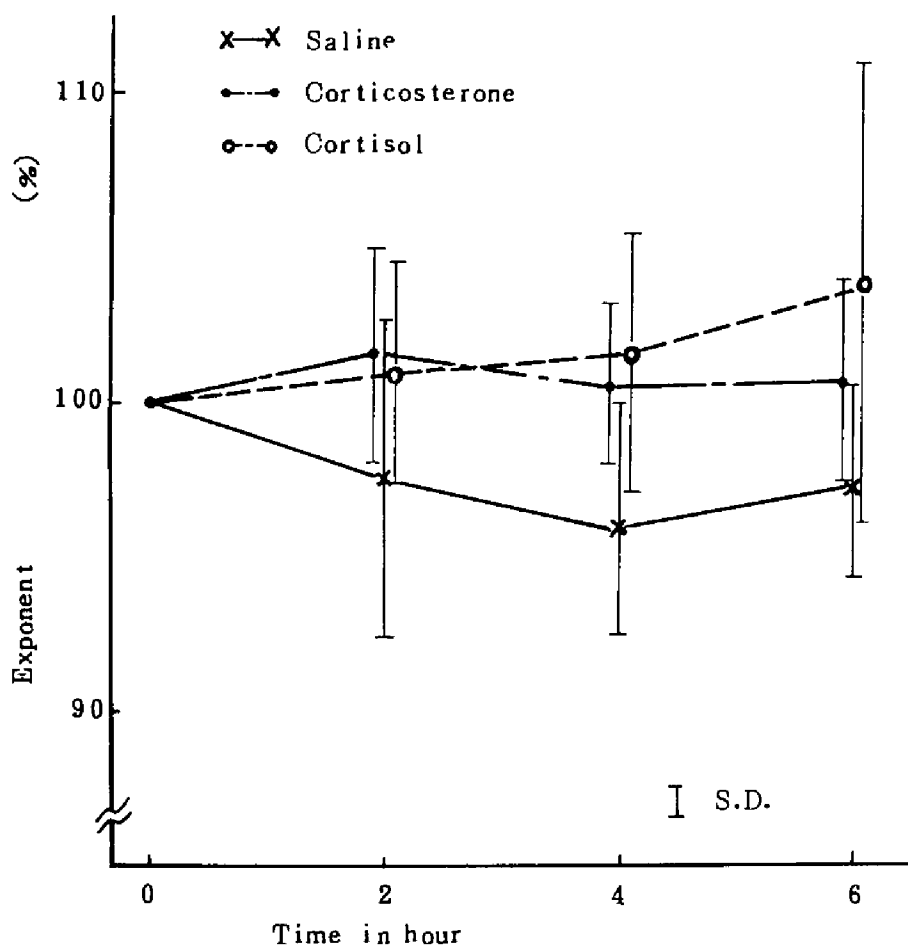


Fig. 31. Influence of an intraperitoneal injection of saline, corticosterone or cortisol on plasma total protein level in sheep

Each value is the mean of six animals

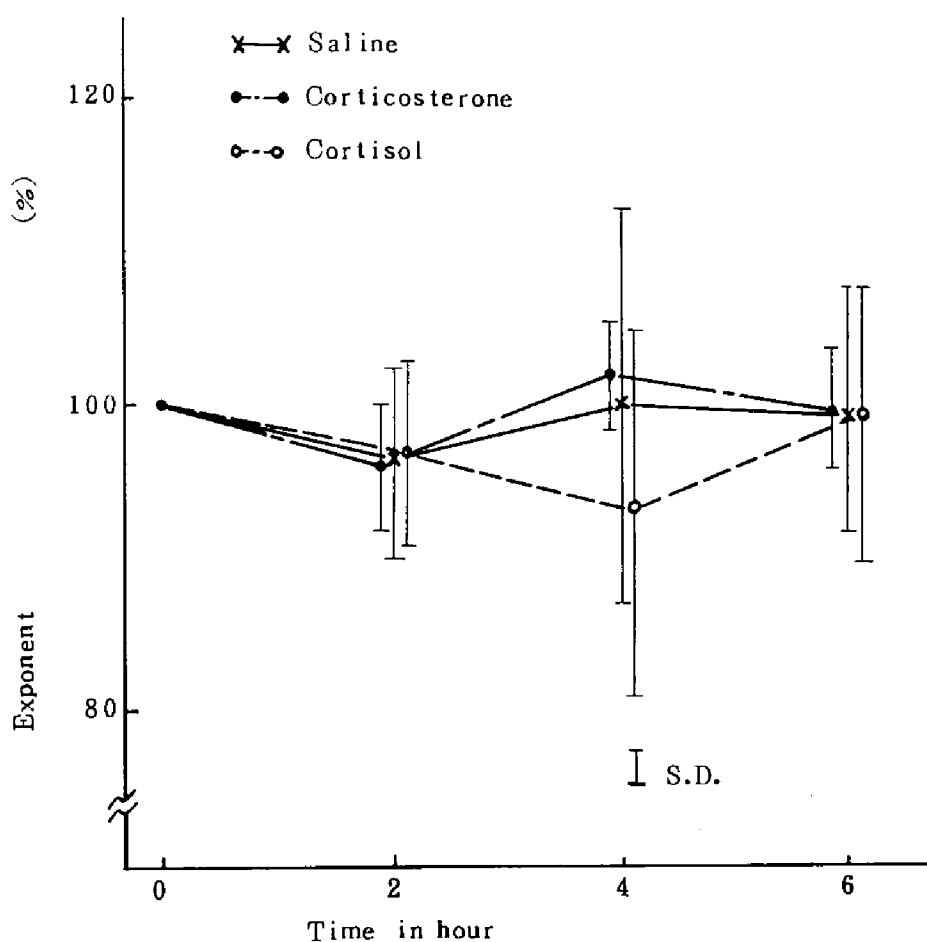


Fig. 32. Influence of an intraperitoneal injection of saline, corticosterone or cortisol on plasma total lipid level in sheep

Each value is the mean of six animals

6 hours after the treatment. The net increases of blood glucose level were 7 % and 38 % in the corticosterone- and cortisol-treated group, respectively (Fig. 30).

As shown in Fig. 31, plasma protein remained constant level in either cortisol- or corticosterone-treated group of sheep, but slightly decreased in the saline-treated. Therefore, both cortisol and corticosterone may have the action to slightly elevate plasma protein level.

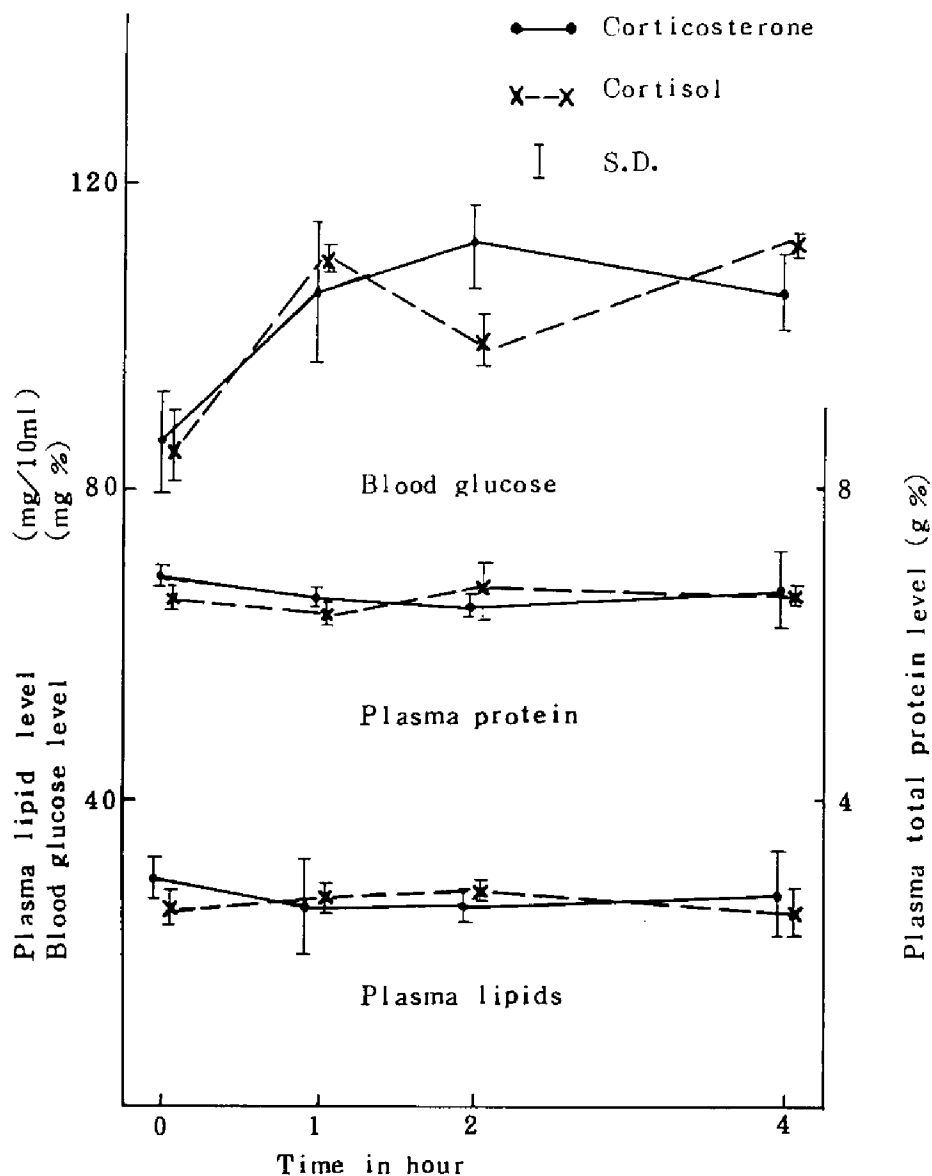


Fig. 33 Influence of an intraperitoneal injection of either corticosterone or cortisol on blood glucose level, and plasma total protein and lipid level in rats

Each value is the mean of two animals

There was no difference in changes of plasma total lipid level following injection of cortisol or corticosterone in sheep (Fig. 32).

As shown in Fig. 33, blood glucose level in rats was rapidly increased by injection of either cortisol or corticosterone ($P < 0.05$), and reached a plateau within one hour after the injection. The increase of the glucose level reached 22 % and 29 % of the initial level in the corticosterone- and cortisol-treated rats, respectively. However, the level prior to the treatment was lower than the previous result (Chapter IV), indicating that the increase in rats might be overestimated. On the other hand, neither plasma protein nor lipid level was changed. Although the number of rats used in this experiment may not be sufficient, the results agreed with the data reported by GLENN *et al.*⁴⁴⁾.

These results suggest that 1) cortisol is more effective for elevation of blood glucose level than corticosterone in both animals, though the difference in rats was only a little, and 2) cortisol is more effective for the elevation in sheep than in rats.

So far as in the present experiment, neither cortisol nor corticosterone affected on plasma total protein and lipid level in both animals. The effect may be related to frequency of the administration of glucocorticoids, based on the reports that consecutive administrations of cortisone increased plasma protein level in rats²²⁾, and that glucocorticoids increased the release of free fatty acids from adipose tissue of rats *in vitro*^{33,34)}.

Chapter XI. Effect of ACTH on growth and food intake

A theory can be proposed from the results obtained hitherto that the higher level of adrenocortical activity is desirable for ruminant growth, but is not always for non-ruminant.

Glucocorticoids increased growth rate in steers^{20,24)} and in wether lambs¹⁴⁰⁾, but decreased the rate in non-ruminant animals^{1,11,12,65,103,104,107,136)}. However, a large amount of exogenous glucocorticoids administered to normal animals may inhibit intrinsic corticotrophin production by the pituitary as reported by LEWIS *et al.*⁷²⁾, indicating that total quantity of the circulating glucocorticoids might be lowered from normal level. Therefore, it was obscure whether the effect on growth was resulted from high adrenocortical activity or not. On the other hand, it has been described that ACTH does not inhibit the intrinsic corticotrophin production regardless of the injections for a long duration^{2,56,159)}.

ACTH was consecutively injected into growing wethers and rats to elevate adrenocortical activity. The effects on growth rate and feed consumption were investigated.

1. Materials and Methods

Male rats (Wistar strain) and wether lambs (Japanese Corriedale), at Animal Breeding Laboratory in Miyazaki University, were used in this study.

This investigation consisted of 4 experiments. ACTH-Z* was used as ACTH preparation. In Exp. I, seventeen rats weighing 265 ± 16 g were divided into 5 groups of 3 each, except for control group having 5 animals. The saline or ACTH was injected intramuscularly into the dorsal buttock region 7 days. Each rat of group 2, 3, 4 and 5 received 0.25, 0.5, 1.0 and 2.0 I.U. of ACTH per head daily, respectively. Body weight was measured at -3, 0, 2, 4 and 7th day from the beginning of each treatment. At the end of this experiment, all rats were

* : ACTH-Z is a commercial preparation of ACTH by Daiichi Seiyaku Co., Ltd. in Japan.

decapitated, and adrenal weights and plasma 11-OHCS levels were measured.

All rats received 1.0 I.U. of ACTH per head daily in Exp. II. The way of injection was the same as in Exp. I. Food intake was determined by subtracting the amount of the remaining from the given food.

These two experiments were conducted in an environment controlled chamber (Koitoiron), where the temperature was set to 25°C and the relative humidity was 50 to 60 % .

The six growing wether lambs (26 — 36 kg body weight) were used commonly in Exps. III and IV. The lambs were daily fed with the concentrate (5 parts of wheat bran and 1 part of soybean meal) at 0.6 % of body weight and alfalfa meal at 0.8 %. Hay and water were given *ad libitum*.

In Exp. III, six lambs were divided into 3 groups of 2 animals each. Each animal in group 1 received the saline as control. Doses of ACTH in groups 2 and 3 were 0.4 I.U. and 0.8 I.U. per kg of body weight per day, respectively. The animals were injected intramuscularly into the dorsal buttock region on day 1, 2 and 3, and again on day 5, 6 and 7 of 15 days experiment. Body weight was measured every 5 days and plasma 11-OHCS levels were determined at 0, 1, 2, 5 and 10 days after the beginning of the treatment.

In Exp. IV, six lambs were divided into 2 groups of 3 animals each. Control group received the saline and the other group received 0.4 I.U. of ACTH per kg of body weight in the same way as in Exp. III. After one month, a treatment for each group was reversed. Body weight was measured 3 consecutive days on every 5 days after the beginning of the treatment.

Plasma 11-OHCS levels were determined by the method described previously (Chapter I).

2. Results

The results of Exp. I are shown in Fig. 34 and Table 26. The injections of saline did not affect the gain of rats. The daily administrations of ACTH inhibited growth over a dose range of 0.25 — 1.0 I.U. per head. A further increase in dose (2.0 I.U. per head) resulted in cessation of growth (Fig. 34). The average daily gain for 7 days decreased

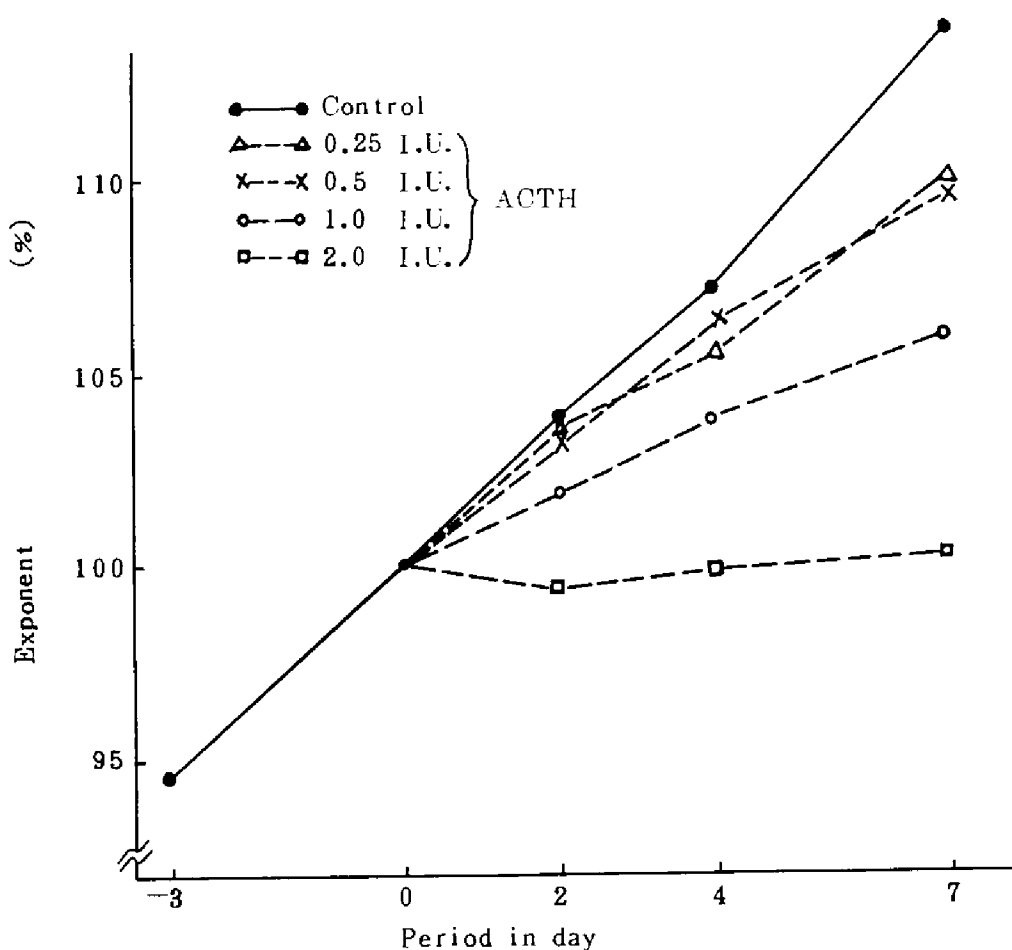


Fig. 34. Effect of intramuscular injections with ACTH on body weight of growing male rats (Exp. I)

progressively as the dose of ACTH increased and ACTH doses more than 1.0 I.U. per head suppressed significantly ($P < 0.01$) the gain comparing with control (Table 26).

Increase of adrenal weight was strictly proportional to the dosage of ACTH ($r = 0.812$, $P < 0.01$). The mean adrenal weight of the animals received either 1.0 or 2.0 I.U. of ACTH was significantly lower than that of controls after 7 days treatment ($P < 0.05$). However, plasma 11-OHCS levels in the ACTH-treated groups were somewhat higher than the levels in the control. Their differences were not significant (Table 26).

As these results, correlation coefficient between growth rate and adrenal weight was -0.79 , and its estimate was statistically significant

Table 26 Effect of intramuscular injections with ACTH on growth rate and adrenocortical activity in growing male rats (Exp. I)

Dose of ACTH (I.U./head/day)	Daily gain (g/day)	Adrenal weight (mg/kg B.W.)	Plasma 11-OHCS level (μ g/100 ml)
0 (5)*	5.4 $\pm$ 1.7 ^{a**}	14.17 $\pm$ 2.13 ^{d**}	9.12 $\pm$ 7.97 ^{**}
0.25 (3)	3.7 $\pm$ 0.4 ^{ab}	17.26 $\pm$ 1.22 ^{de}	15.15 $\pm$ 4.24
0.5 (3)	3.8 $\pm$ 1.0 ^{ab}	18.06 $\pm$ 3.34 ^{de}	13.43 $\pm$ 5.85
1.0 (3)	2.2 $\pm$ 1.1 ^{bc}	23.71 $\pm$ 3.75 ^{ef}	13.37 $\pm$ 1.33
2.0 (3)	0.1 $\pm$ 0.9 ^c	29.77 $\pm$ 9.72 ^f	20.84 $\pm$ 5.32

a, b and c: Differences between means with the different letter are significant ($P < 0.01$)

d, e and f: Differences between means with the different letter are significant ($P < 0.05$)

*: The number in the parentheses shows a number of animals

** : Mean $\pm$ standard deviation

($P < 0.01$).

In Exp. II, the injections of 1.0 I.U. of ACTH inhibited growth and feed consumption immediately after the beginning of the treatment in rats (Fig. 35). Moreover, feeds required for a unit (= g) of gain, i.e., feed conversion ratio, were 6.4 g and 15.1 g in controls and in treatments, respectively.

Exp. III was conducted as the preliminary experiment on sheep. The average daily gains of three groups received saline, 0.4 I.U. and 0.8 I.U. of ACTH per kg of body weight, were 107 g, 144 g and 128 g, respectively. Plasma 11-OHCS levels in ACTH-treated groups were somewhat higher than those in the control.

The results of Exp. IV are shown in Figs. 36 and 37. Growth rate for 10 days after the beginning of the treatment was higher in ACTH-treated sheep than in the control, though it was not significantly effective.

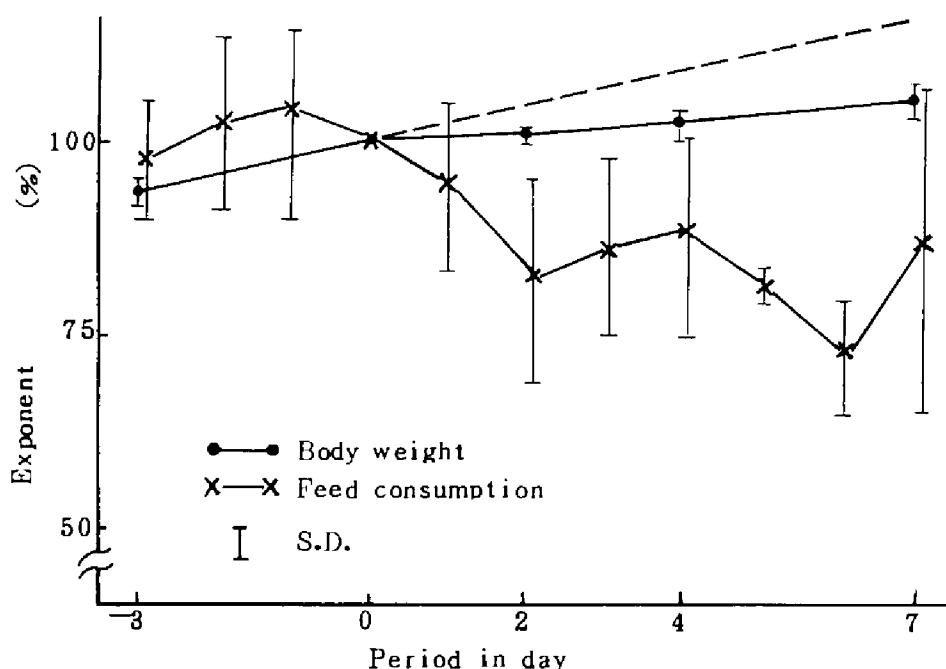


Fig. 35. Effect of intramuscular injections with ACTH on body weight and feed consumption of growing male rats (Exp. II)

It was not understood why growth rate during 5 to 10 days was higher than the rest of the periods in both groups (Fig. 36). Total gains for these 10 days were 0.64 kg and 1.00 kg in the saline- and ACTH-treated group, respectively. There was an increase in the gain of 56 % in the ACTH-treated wethers. This difference was not statistically significant. However, it could be interpreted that ACTH accelerated the growth in wethers, since the gain following ACTH treatment was higher than that following saline treatment in all sheep except for only one animal (No. 2)(Table 27). Moreover, while roughage consumption was constant in the saline-treated group, it tended to increase in ACTH-treated (Fig. 37). Roughage volumes required for a unit (= kg) of gain were 8.9 kg and more than 13.5 kg in treatments and in controls, respectively.

3 Discussion

ACTH is likely to stimulate durable production of glucocorticoids.

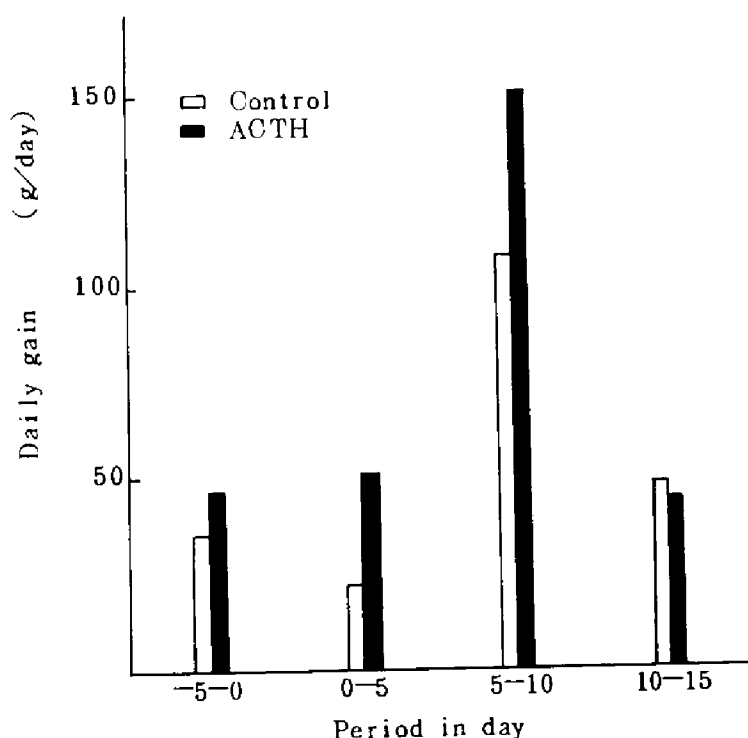


Fig. 36. Effect of intramuscular injections with ACTH on daily gain of growing wether lambs (Exp. IV)

The animals were injected intramuscularly with ACTH (0.4 I.U./kg of body weight) on day 1, 2 and 3, and again on day 5, 6 and 7 of 15 days experiment.

ACTH somewhat increased plasma 11-OHCS levels, but the levels did not increase in parallel with the doses in rats and sheep. On the other hand, adrenal weights were highly correlated with doses of ACTH in rats ($P < 0.01$). These results indicate that weight of the adrenal is a good index of adrenocortical activity in experiments of more than a few day duration as suggested by SAYERS¹²⁸).

In rats growth rate was decreased when the adrenocortical activity was stimulated by ACTH. This agreed with the results in rats^{11,103,104}), chickens^{12,136}) and rabbits⁶⁵) with injections of glucocorticoids. On the contrary, there was more increase in the gain of 56 % in the wether lambs injected ACTH than in the controls. The elevated growth rate in wethers seemed to result from a high adrenocortical activity, though no

Table 27 Total gain for ten days after the beginning of either ACTH or saline treatment in sheep

Sheep No.	Gain (kg)	
	Saline	ACTH
1	-0.27	0.40
2	1.62	0.70
3	0.75	1.12
4	0.23	0.68
5	-0.01	1.37
6	1.51	1.73
Mean	0.64	1.00

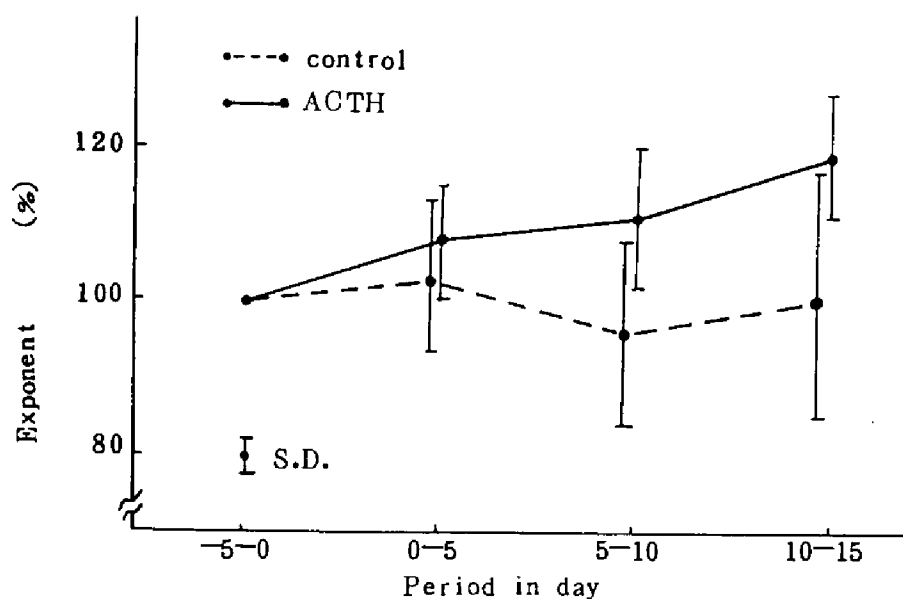


Fig. 37 Effect of intramuscular injections with ACTH on feed consumption of growing wether lambs (Exp. IV)

The animals were injected intramuscularly with ACTH (0.4 IU/kg of body weight) on day 1, 2 and 3, and again on day 5, 6 and 7 of 15 days experiment.

direct evidence that the consecutive ACTH injections kept the activity elevating was obtained in this study.

It is generally accepted that glucocorticoids accelerate the protein catabolism in chickens¹²⁾, rats¹¹⁹⁾ and sheep⁸⁸⁾, or make protein synthesis retard in mice⁴⁾ and rats²⁵⁾. One of the causes of the different growth response to ACTH between ruminants and rodents might be feed consumption; the consumption decreased in rats after ACTH injection, but increased in sheep. Another might be efficiency of feed utilization; the efficiency decreased in rats after ACTH injection, but increased in sheep.

When adrenocortical activity was stimulated by the consecutive injections of ACTH, as a whole, growth rate decreased in rats, whereas it increased in sheep. This result would give further information to support the theory postulated above.

Summary and Conclusion

Physiological aspects of the digestion and metabolism in ruminants are principally different from those in monogastric animals. The author supposed that the adrenocortical function of ruminants might adapt itself to the nutritional metabolism. The specificity of adrenal cortex in ruminants was investigated by means of comparative approach, compared with that in non-ruminant animals.

I. Studies on establishment of analytical method for glucocorticoids (11-OHCS)

Analytical methods for 11-OHCS were investigated with blood plasma and adrenal tissue samples of domestic and laboratory animals.

- 1) Quantitative methods for 11-OHCS level in both plasma and adrenal tissue were established in this study. Recoveries of cortisol and corticosterone added to plasma were $97.4 \pm 9.0 \%$ and $101.2 \pm 10.4 \%$, respectively. Recoveries of cortisol and corticosterone added to adrenal tissue were $85.3 \pm 11.1 \%$ and $108.0 \pm 12.0 \%$, respectively.
- 2) A method with thin layer chromatography for determination of a ratio of cortisol to corticosterone (F/B ratio) was also established.
- 3) Some evidences indicated appropriateness of the obtained methods.

II. Adrenal weights in various animals

The wet adrenal weights were compared among animal species; cattle (Japanese Black and Holstein), sheep (Japanese Corriedale), rabbits (Japanese native), domestic fowls (White Leghorn) and rats (Wistar strain).

The order of the magnitude on the adrenal weight per unit of body weight was as follows; cattle < sheep < domestic fowls < rabbits < rats. However, when the adrenal weight was expressed as a function of metabolic body weight ($W^{3/4}$), the differences among species were not evident. It is supposed therefore that the adrenal glands of cattle and sheep may be as important as those of other animals, functionally.

III. 11-OHCS levels in both blood plasma and adrenal tissue of various animals

To elucidate the specificity of adrenocortical function in ruminants, 11-OHCS levels and F/B ratios in both plasma and adrenal tissue were compared among various animals. The used animals are the same as in Chapter II.

- 1) The levels of 11-OHCS in plasma of ruminants were lower (under 4 $\mu\text{g}/100\text{ml}$) than those in all other animals.
- 2) The levels of 11-OHCS in adrenal glands of cattle and sheep were higher than those of rabbits and rats. But, this might be simply due to the conditions under which the adrenal glands were taken out.
- 3) The F/B ratio in plasma of ruminants was over 3.0, indicated that cortisol was a predominant corticoid in the plasma.

IV. Effect of season, age and sex on plasma 11-OHCS level

Blood samples of cattle, sheep and rats collected during early summer or late winter were analyzed to elucidate the influence of season, age and sex on plasma 11-OHCS level. The used animals consisted of both sexes of various ages in each species.

- 1) Plasma 11-OHCS levels of the female were higher than those of the male in cattle and rats, while the sexual difference was obscure in sheep.
- 2) In calves seven to twelve months old, plasma 11-OHCS levels were high in winter and low in summer. Similar seasonal effect was observed in rats, but not in sheep.
- 3) Influence of age on plasma 11-OHCS levels was appreciable in sheep and rats.

V. Effect of physical and psychological stresses on plasma 11-OHCS level

The effects of meteorological and transportation stressors on plasma 11-OHCS level were investigated in sheep, rabbits and rats.

- 1) Plasma 11-OHCS level increased in sheep and rats by "hot" stress. This response was remarkably significant in rats ($P < 0.01$).

- 2) Plasma 11-OHCS level increased significantly in rats by 'cold' stress ($P < 0.05$), but the level somewhat decreased in sheep.
- 3) In general, the responses of plasma 11-OHCS level to physical stresses in sheep were less than those in rats.
- 4) Plasma 11-OHCS level was increased by transportation stressor in all animal species, especially in rabbits.

VI. Effect of fasting on plasma 11-OHCS level

The response of plasma 11-OHCS level and adrenal weight to fasting was investigated in sheep, rabbits and rats.

- 1) Plasma 11-OHCS level in sheep increased significantly ($P < 0.05$) within 10 days and elevated moreover at 20th to 24th day after the removal of feeds. The levels decreased suddenly prior to death.
- 2) Plasma 11-OHCS levels in rabbits were rather low at the early stage of fasting than the initial levels, but somewhat increased at 10th day after the beginning of fasting.
- 3) Plasma 11-OHCS levels in rats were nonsignificantly affected by fasting for 7 days.
- 4) Adrenal glands of sheep died of fasting were hypertrophic, but those of rats were not affected.

VII. Role of glucocorticoids on glucose metabolism in fasting animals

Changes of blood glucose, protein and lipid level during fasting in sheep and rats, and the effects of glucose injection into the fasted sheep on plasma 11-OHCS, protein and lipid level were studied.

- 1) Blood glucose level dramatically decreased at first ($P < 0.05$), but the lowered level was maintained or even somewhat elevated thereafter through fasting in sheep and rats.
- 2) Plasma total protein level gradually increased up to 10 days of fasting in sheep ($P < 0.01$), but began to decrease from 4th day after the removal of feeds in rats and its decrease became significant at 6th day in female ($P < 0.05$).
- 3) The similar changes as in protein were observed in plasma total

lipid level in both species ($P < 0.05$). The results of lipids in sheep seemed to be primarily due to the increased free fatty acids (FFAs) ($P < 0.01$).

4) The intravenous glucose injection reduced plasma 11-OHCS level in the fasted sheep ($P < 0.01$).

5) After the glucose injection, plasma total protein levels decreased ($P < 0.01$) and the lipid levels decreased ($P < 0.05$), most likely by declining of FFA content ($P < 0.01$).

6) Glucocorticoids seemed to play more important role for the maintenance of blood glucose level during fasting in sheep than in rats.

VIII. Effect of ACTH on plasma 11-OHCS level and F/B ratio

The responses of plasma 11-OHCS level and F/B ratio in blood plasma to an intraperitoneal injection of ACTH were studied in cattle, sheep, rabbits and rats.

1) ACTH injection caused a transient increase of plasma 11-OHCS level. It took two hours in cattle and sheep, one hour in rabbits and thirty minutes in rats to reach the maximum level.

2) The time required to reach the maximum plasma 11-OHCS level in sheep was constantly two hours regardless of the various ACTH doses.

3) The percentages of plasma 11-OHCS after the ACTH injection to the initial levels were 582 in cattle, 326 in sheep, 381 in rabbits and 121 in rats ($P < 0.05$). But the difference between sheep and rabbits was not statistically significant.

4) There was a tendency that F/B ratio in plasma increased in cattle and sheep after the ACTH injection, while the ratio decreased in rabbits and rats.

IX. Effect of ACTH, cortisol and corticosterone injection on blood eosinophils count

The responses of eosinophils count in the peripheral blood to an intraperitoneal injection of ACTH, cortisol or corticosterone were investigated in cattle, sheep, rabbits and rats.

1) Eosinophils count dramatically decreased in both cattle and sheep

after the ACTH injection ($P < 0.01$), but the count increased in rabbits ($P < 0.05$) and was not changed in rats.

2) Eosinophils count decreased by both cortisol and corticosterone in either case of sheep or rats. Especially, the effect of cortisol was greater than that of corticosterone.

3) Eosinopenia was also induced in sheep, even if the dose of cortisol was reduced to about one third of the previous case, while it was not in rats, when the dose was reduced to about one half.

X. Effect of cortisol and corticosterone on blood glucose, protein and lipid level

The effects of an intraperitoneal injection of cortisol and corticosterone on blood glucose, plasma protein and lipid level were tested in sheep and rats.

1) The net increases of blood glucose level were 7 % and 38 % in the corticosterone- and cortisol-treated sheep, respectively, up to 6 hours after the injection.

2) The increases of blood glucose level were 22 % and 29 % of the initial level in the corticosterone- and cortisol-treated rats, respectively.

3) No difference was distinctly appreciated in regard to changes of plasma total protein and lipid level after the injection of either cortisol or corticosterone.

4) As a conclusion of Chapters IX and X, there seems to be a difference in activity between cortisol and corticosterone, and in receptivity between sheep and rats regarding the effects of the hormones on peripheral blood eosinophils count and blood glucose level.

XI. Effect of ACTH on growth and food intake

The effect of intramuscular injections of ACTH-Z on growth and food intake was investigated in growing wethers and male rats.

1) Growth rate and feed consumption significantly decreased ($P < 0.01$) in rats injected with ACTH, while they increased in sheep.

2) Feed conversion ratio in ACTH-treated sheep was lower than that in control, while it was the reverse in rats.

3) The different response of growth rate to ACTH between ruminants and rodents seems to be made part of feed consumption and part of efficiency of feed utilization.

From the above results, specificities of adrenocortical function related to ruminant nutrition are summarized as follows;

- 1) The predominant corticoid in plasma of ruminants was cortisol.
- 2) Plasma 11-OHCS level responded more sensitively to fasting in sheep than in rabbits and rats.
- 3) Glucocorticoids played an important role in the mechanism for the maintenance of blood glucose level during fasting in sheep.
- 4) Response of adrenal cortex to ACTH was higher in cattle and sheep than in rats.
- 5) Cortisol was more effective for elevation of blood glucose level than corticosterone in both sheep and rats, and the receptivity to cortisol was higher in sheep than in rats.
- 6) The higher level of adrenocortical activity was desirable for ruminant growth, but not always for non-ruminant.

In general, glucose is essential for nervous conductivity in mammals^{55,67,157}). It depends on the level in blood glucose whether the supply to brain, central nervous system, etc. is sufficient or not⁸⁰). However, only little part of carbohydrate in feeds is absorbed as glucose from gut in ruminants^{3,52,74}), having the low blood glucose level even normally. Therefore, even a little shortage of glucose could be a growth limiting factor in ruminants.

As described previously (1~5) , the adrenal cortex in ruminants had some specificities advantageous to compensate the growth limiting factor, suggesting that gluconeogenetic action of glucocorticoids was of particular importance in them.

If glucocorticoids stimulate simply and solely the gluconeogenesis from amino acids in ruminants as in non-ruminants¹⁵⁵), their action would be catabolic and they would inhibit the growth. However, it is the most unique characteristic of ruminant nutrition that a great amount of volatile fatty acids are absorbed from gut, converted to glucose, ketone body, etc. and utilized in body.

Supposing that glucocorticoids stimulate the gluconeogenesis from

the volatile fatty acids, glucose will be supplied sufficiently in ruminants. It would solve the problem of unstable glucose nutrition without protein catabolism and also would make the utilization of the fatty acids efficient. It would increase the feed intake, moreover, because the accumulation of the fatty acids decreased the intake⁹⁾. As the results, hyperadrenocortical activity increased the growth rate in ruminants (6)).

As a whole, it can be concluded that the unique characteristics of adrenal cortex in ruminants should be the result from the adaptation to the peculiarity of metabolism of nutrients, particularly carbohydrate metabolism.

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