Journal of Steroid Biochemistry, 1972, Vol. 3, pp. 907–917. Pergamon Press. Printed in Great Britain

STUDIES ON SECRETION MECHANISM OF ADRENAL CORTICOSTERONE: ADRENAL SUBCELLULAR DISTRIBUTION OF CORTICOSTERONE AND ITS CHANGES ON HYPOPHYSECTOMY AND ACTH ADMINISTRATION*

M. INABA, K. KAMATA, S. IMAI† and T. NAKAO†
Division of Bio-Function Research, Biomedical Research Laboratories and Department of Pharmacology†, Jikei University School of Medicine, Minato-ku, Tokyo 105, Japan.

(*Received* 17 *April* 1972)

SUMMARY

With intact, hypophysectomized and ACTH-administered hypophysectomized rats, intracellular distribution of adrenal corticosterone was studied by determining this steroid in each of the nuclear (800 *g* pellet), mitochondrial (12,000 *g* pellet), microsomal (105,000 *g* pellet) and cytosol (105,000 *g* supernatant) fractions. The proportion of corticosterone in the microsomal fraction as compared with the whole adrenal corticosterone became greater in the hypophysectomized rats and smaller in ACTH-treated, hypophysectomized rats than in the intact rats. Corticosterone content of the microsomal fraction was slightly altered on hypophysectomy and ACTH administration. The corticosterone content of the nuclear fraction was decreased on hypophysectomy and increased remarkably on ACTH administration. The proportion of corticosterone in the nuclear fraction to corticosterone in the whole intracellular organellae of adrenal showed opposite changes to those observed with microsomal corticosterone on hypophysectomy and ACTH administration.

Non-dialyzable corticosterone was found present in the adrenal cytoplasmic fraction by the dialysis experiments.

A mechanism of secretion of corticosterone was discussed from a point of view on intracellular distribution of this steroid and its changes on hypophysectomy and ACTH administration.

INTRODUCTION

IT IS WELL known that synthesis and secretion of corticosterone is a major function of the rat adrenal cortex. Although much has been done to elucidate the sequential reactions of steroidogenesis in adrenocortical cells in relation to the mechanism of ACTH action, little attention has been paid to the mechanism of the hormone secretion. It is usually assumed that corticosterone biosynthesized as one of the end products in the adrenal mitochondria will be released into cytoplasm in a free form, then transported to the outside of the adrenal cell by simple diffusion due to differences between intracellular and extracellular corticosterone concentrations. This may be true, but also may not be the whole procedure involved in the adrenal corticosterone secretion.

The present experiments were carried out to study subcellular distribution of the rat adrenal corticosterone, its changes due to hypophysectomy and ACTH administration, and a possible pre-secretory form of corticosterone in the rat adrenal.

*Presented in part at the Annual Meeting of Japan Endocrinological Society, April 1972, Tokyo, Japan.

MATERIALS AND METHODS

Biological material and treatment. Male rats of the Donryu strain, supplied by Japan Rat Co., Urawa City, Japan, were housed at a temperature (23–24°C) and illumination (6.00 a.m.–6.00 p.m.) controlled animal room and fed with a standard laboratory chow (CE-2, Japan CLEA Co., Tokyo) and water *ad libitum*. Handling of the rats was done by means of weighing them every day soon after they were housed. In order that the rats be accustomed to injection they were given 0·1 ml of saline (0·9% NaCl) by subcutaneous injection regularly at a definite time (10.00–10.30 a.m.) every day for at least 7 days until they were killed for experiment. Adult rats were used for experiment when they were weighing approx. 200 g.

Injection of ACTH and hypophysectomy. One experimental group of rats usually consisted of 5 rats of the same age. ACTH (Schering) was injected subcutaneously in a dose of 1 unit/rat in saline at 10 a.m. and 30 min before decapitation. The control rats were given 1 ml of saline alone. To avoid circadian variations, a time table for injection of ACTH or saline into intact rats as well as decapitation was strictly adhered to. Hypophysectomy was performed by the parapharyngeal route under pentobarbital sodium anesthesia (30 mg/kg body weight intraperitoneally), and the hypophysectomized rats (hypox rats) were subjected to experiment 20–23 h after operation.

Adrenal tissue fractionation. To obtain adrenal subcellular fractions, 10 adrenals from 5 rats in one group were taken out onto a filter paper moistened with saline as soon as possible after they were decapitated, freed of adhering fat and weighed on a torsion balance. They were then homogenized in 2 ml of cold 0·25 M sucrose with a tight-fitting all glass homogenizer of Potter–Elvehjiem type, by 3–4 gentle strokes at 2–3°C. The adrenal homogenate combined with washes of the homogenizer (twice with 0·5 ml of cold 0·25 M sucrose) was then centrifuged at 800 *g* for 10 min at 2°C in a Tomy NO-90UV refrigerated centrifuge (Tomy Instrument Co. Ltd., Tokyo). The 800 *g* pellet, called the nuclear fraction, was washed twice by resuspending and recentrifuging in the homogenization buffer. The 800 *g* supernatant fraction was centrifuged at 12,000 *g* for 30 min at 2°C. The pellet, called the mitochondrial fraction, was washed twice. The 12,000 *g* supernatant fraction was centrifuged at 105,000 *g* for 1 h at 2°C in a Hitachi model 55PA ultracentrifuge equipped with a RP55A rotor (Hitachi, Ltd., Tokyo). The pellet, referred to as the microsomal fraction, was washed twice. The 105,000 *g* supernatant fraction was called the cytosol fraction. Each pellet was added 2 ml of distilled water, mixed thoroughly and kept in a refrigerator overnight.

Corticosterone determination. For determination of serum corticosterone, peripheral blood was collected in a conical centrifuge tube from a decapitated rat and centrifuged at 3,000 rev./min for 30 min. Serum corticosterone was determined by the method of Guillemin *et al.* [1] which was partially modified. To avoid formation of emulsion in extraction of serum with dichloromethane and to make the blank values lower, the whole procedure was performed at 3–5°C in contrast with the original method which was performed at room temperature. For determination of corticosterone content in a subcellular fraction, 2 aliquots of a waterlyzed suspension of the pellet in one tenth volume were placed in conical tubes for determination of protein and to the remainder was added distilled water to make the volume 5 ml for determination of corticosterone. Protein was deter-

mined in duplicate by the method of Lowry *et al.*[2]. An Aminco-Bowman fluorospectrophotometer (American Instrument Company, Inc., Maryland, U.S.A.) was employed for fluorometric determination of corticosterone. A criterion for determining corticosterone in the adrenal subcellular fractions was the coincidence of the fluorescence spectra of the determined substances in the fractions with that of the standard corticosterone (Fig. 1).

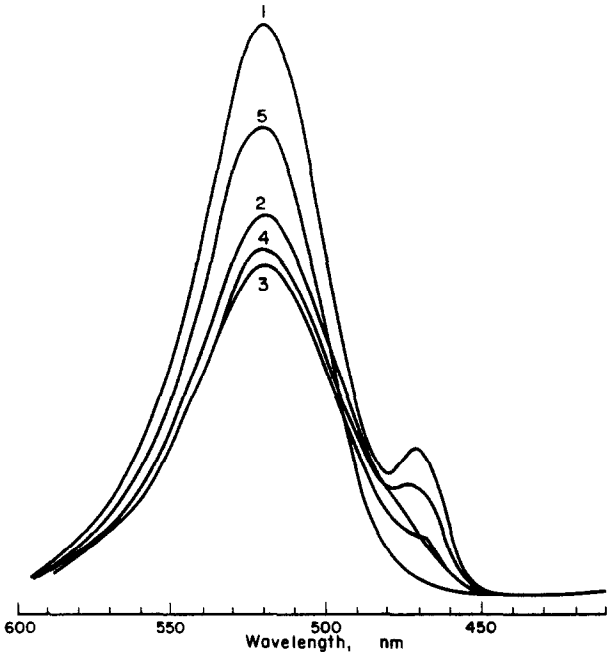

Fig. 1. Fluorescence spectra of the determined substance(s) in adrenal subcellular fractions by the method of Guillemine *et al.* which was partially modified and the spectrum of the standard corticosterone. Fluorescence spectrum of (1) the standard corticosterone, (2) nuclear (800 *g* pellet) fraction, (3) mitochondrial (12,000 *g* pellet) fraction, (4) microsomal (105,000 *g* pellet) fraction, (5) cytosol (105,000 *g* supernatant) fraction. Excitation wavelength: 470 nm. See text for the details of partial modification of the method of Guillemin *et al.*[1].

Dialysis experiment. One ml of the mixture of adrenal microsomal and cytosol fraction (post-mitochondrial fraction in Table 2) was put in a dialysis tube (Visking seamless cellulose tubing, 6·4 m/m) and dialyzed against the 5 volumes of distilled water or saline at 22–23°C for 24 h. The cytosol fraction was similarly dialyzed. In a continuous dialysis, the successive dialysis was performed for several days by replacing the outer liquid with the same volume of distilled water or saline every 24 or 48 h in a cold room at 3°C. Quantities of corticosterone in the outer liquids as well as inside the tube were determined after completion of dialysis.

RESULTS

Fluorescence spectra of determined substances in adrenal subcellular fractions

The adrenal subcellular fractions were subjected to determination of corticosterone. Fluorescence spectra of the substances determined in the fractions were studied with a maximal excitation wavelength of 470 nm in the fluorospectrophotometer. As shown in Fig. 1, all spectra of the adrenal subcellular fractions coincided well with the spectrum of standard corticosterone (Fig. 1).

Changes in serum and adrenal corticosterone on hypophysectomy and ACTH administration

The concentration of corticosterone in serum and in adrenal cytosol was determined with intact rats, hypox rats and hypox rats given 1 unit of ACTH. As seen in Table 1, the serum corticosterone concentration in the hypox rats was

Table 1. Changes in concentration of serum corticosterone and content of adrenal cytosol corticosterone as well as organella corticosterone on hypophysectomy and ACTH administration

Rats	(No. of Rats)	Serum Comp.B** (μg/dl serum)	Cytosol Comp.B** (μg/100 mg protein)	Organella Comp.B** (μg/100 mg protein)
Intact	(5)	9·95 ± 0·61*	60·05***	12·69***
Hypox†	(5)	1·20 ± 0·31*	20·17	3·98
ACTH administered Hypox‡	(5)	31·08 ± 5·42*	140·73	7·55

*Mean ± S.D.

**Corticosterone, a criterion on which the determined substance is recognized to be corticosterone was based on a coincidence of the fluorescence spectrum with that of the standard corticosterone.

***Average of the duplicate determinations, adrenal cytosol and organella fractions were prepared by stepwise centrifugations of 0·25 M sucrose homogenate of 10 adrenals from 5 rats in one group (see text for the details).

†Hypox rats, hypophysectomized rats 20–23 h after the operation.

‡Hypophysectomized rats 20–23 h after the operation which received administration of 1 unit ACTH 30 min prior to decapitation.

1·20 ± 0·31 μg/100 ml (Mean ± S.D., n = number of rats used 5) which was about one eighth of that in the intact rats (9·95 ± 0·61 μg/100 ml, n = 5). Thirty min after ACTH administration to the hypox rats, it was elevated as much as 26 times than that of control hypox rats injected saline instead of ACTH. On the other hand, corticosterone in the cytosol fluctuated to a lesser extent than in the serum on hypophysectomy and ACTH administration. The corticosterone content of the cytosol of hypox rats was 20 μg/100 mg protein, which was approx. one third of that with intact rats (60 μg/100 mg protein). ACTH administration to hypox rats elevated the cytosol corticosterone content to 140 μg/100 mg protein, but the 7-fold increment was considerably lower than the 26-fold increment in serum corticosterone concentration on ACTH administration. Protein content of the cytosol fraction did not change on hypophysectomy and ACTH administration. A mixture of nuclear, mitochondrial and microsomal fractions is defined as the adrenal organella fraction. On hypophysectomy, the corticosterone content of the organella fraction was decreased to the same extent as in cytoplasmic fraction, but the extent of its increase on ACTH administration was considerably less than in cytoplasmic fraction (Table 1). In summary, it is evident that fluctuation of serum corticosterone concentration due to hypophysectomy or ACTH administration is much greater than that of corticosterone content in cytoplasmic fraction. On ACTH administration to hypox rats, the extent of increase in corticosterone concentration is in the following order; 26-fold in serum > 7-fold in cytoplasmic fraction > 2-fold in organella fraction. The decrease in serum corti-

costerone due to hypophysectomy is much greater than that of corticosterone content of the adrenal cytoplasmic fraction.

Changes in subcellular distribution of adrenal corticosterone on hypophysectomy and ACTH administration

Corticosterone contents (μg/100 mg protein) of the 4 subcellular fractions of the adrenals of intact, hypox and ACTH-administered hypox rats are shown in Fig. 2. The highest content of corticosterone was observed with the cytosol

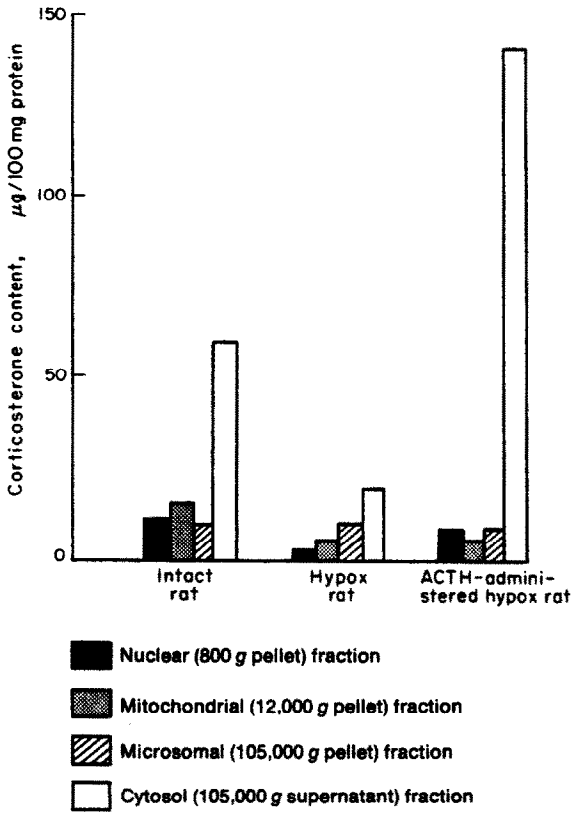

Fig. 2. Corticosterone contents in adrenal subcellular fractions of intact, hypox and ACTH-administered hypox rats.

fractions in all of the rats in the 3 groups. A clear decrease of corticosterone content on hypophysectomy as well as a remarkable increase of that on ACTH administration was previously described for the cytosol fraction which was mostly extensively influenced by hypophysectomy and ACTH administration among the 4 subcellular fractions. It is interesting to note that the corticosterone content of the nuclear fraction was changed by hypophysectomy and ACTH administration in the same manner as that of the cytoplasmic fraction. The corticosterone content of mitochondria was decreased by hypophysectomy but it was not restored by ACTH administration. The corticosterone content in the microsomal fraction was almost unchanged in the adrenals of intact, hypox and ACTH-administered hypox rats.

The proportion of corticosterone in each subcellular fraction to the total amount of corticosterone in the whole adrenal was expressed as a percentage. The % distributions of adrenal subcellular corticosterone with intact, hypox and ACTH-administered hypox rats are illustrated in Fig. 3. It is noticed that the

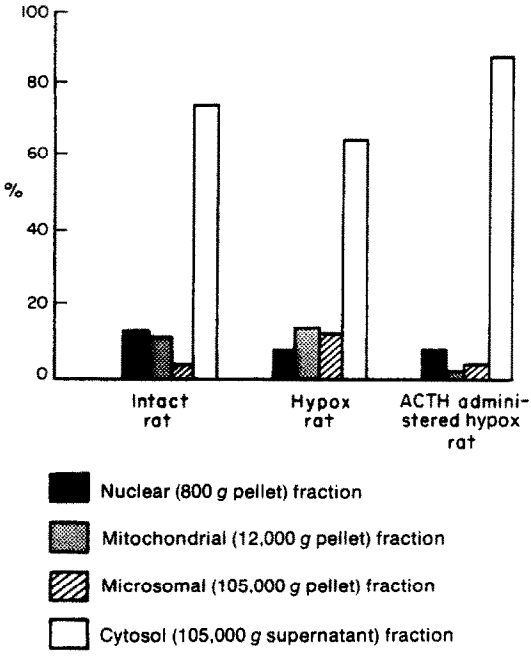

Fig. 3. Distributions of intracellular corticosterone in adrenals of intact, hypophysectomized and ACTH administered rats.

proportion of cytosol corticosterone was kept relatively constant though a slight drop was observed on hypophysectomy and a moderate elevation followed ACTH administration. Although almost all corticosterone in the adrenal was located in the cytosol fraction, the fairly large proportions were found with the mitochondrial and microsomal fraction of hypox rats. It is interesting to note that the proportion of the microsomal corticosterone was increased remarkably by hypophysectomy and decreased markedly on ACTH administration. Localization of corticosterone was noticed in the nuclear fractions with considerable proportions in all the cases. As the nuclear fraction contains the large fragments of adrenal cell membrane other than nuclei, the determined substance with the 800 *g* particle is supposedly attributed to corticosterone associated with the adrenal cell membrane. Proportions of the amounts of corticosterone in the nuclear, mitochondrial and microsomal fraction to the total amount of corticosterone in the adrenal organella fraction were also expressed in percentages. The % distribution of intraorganella corticosterone in intact, hypox and ACTH-administered hypox rats is illustrated in Fig. 4. It was again recognized that the percentage of nuclear corticosterone was decreased by hypophysectomy and remarkably increased on ACTH administration. Increase in the % of corticosterone in the microsomal fraction on hypophysectomy and decrease on ACTH administration were again clearly seen in this type of corticosterone distribution.

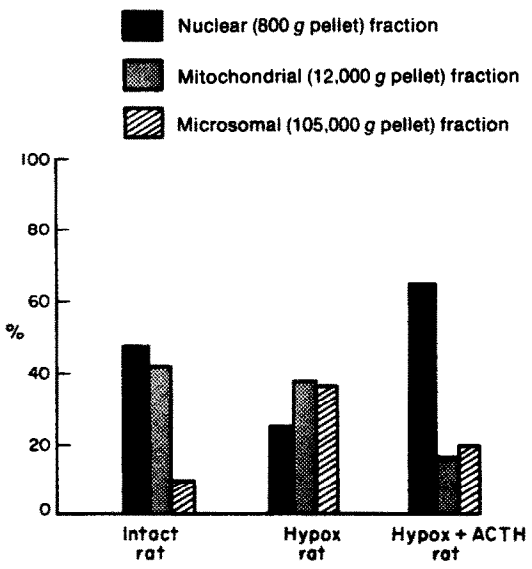

Fig. 4. Intraorganella distributions of corticosterone in adrenals of intact, hypophysectomized and ACTH-administered hypophysectomized rats.

In short, both intracellular and intraorganella distribution of the adrenal corticosterone were affected by hypophysectomy and ACTH administration. The most striking change in the intracellular and intraorganella distribution was observed with the microsomal and nuclear fractions.

Dialysis of adrenal microsomal and cytosol fraction

To investigate the possibility that intracellular corticosterone is not only in a free form but also in a bound form which might play a significant role in intracellular movement of the hormone, dialysis experiments were carried out with the mixture of adrenal microsomes and cytosol (post-mitochondrial fraction). A dialysis tube containing 1·0 μg of corticosterone in 1·0 ml of 0·25 M sucrose was put in a test tube which contained 5·0 ml of distilled water or saline and kept for 24 h at room temperature (22–23°C). As seen in Table 2, the ratios of corticosterone in the inner liquid to that in the outer liquid were 1 : 4·8 and 1 : 4·3 after 24 h duration of dialysis, indicating that an equilibrium of free corticosterone between the inner and outer liquid was almost achieved after 24 h dialysis at room temperature. Under the same condition, the 2 post-mitochondrial fractions obtained by centrifugation of 0·25 M sucrose homogenate of 96 mg adrenal (6 adrenals from 3 intact rats weighing 375–475 g) at 12,000 *g* for 30 min at 2°C were dialyzed against distilled water and saline separately for 24 h. As seen in Table 2, the ratios obtained were 1 : 1·2 and 1 : 1·4 respectively, indicating that a non-dialyzable corticosterone was contained in the adrenal microsomal fraction or cytosol fraction. The same type of dialysis experiment was carried out with the adrenal cytosol fraction, and the ratios obtained were 1 : 1·7 and 1 : 2·1, indicating that a non dialyzable corticosterone was contained in the adrenal cytoplasmic fraction. A continuous dialysis of the mixed fraction of microsomes and cytosol

Table 2. Dialysis of a mixture of adrenal microsomal and cytosol fraction (post-mitochondrial fraction) and dialysis of adrenal cytosol fraction

Liquid for dialysis		Volume of liquid	Corticosterone found(μg)	ratios
Inner(Inn) Comp.B in sucrose*		1 ml	0·14	$\frac{1}{4·8}$
Outer(Out) 0·9% NaCl		5 ml	0·67	
Inn.	Comp.B in sucrose*	1 ml	0·15	$\frac{1}{4·3}$
Out.	Distilled water	5 ml	0·65	
Inn.	Post-Mt fraction**	1 ml	0·21	$\frac{1}{1·4}$
Out.	0·9% NaCl	5 ml	0·31	
Inn.	Post-Mt fraction	1 ml	0·25	$\frac{1}{1·2}$
Out.	Distilled water	5 ml	0·31	
Inn.	Cytosol fraction	1 ml	0·23	$\frac{1}{1·7}$
Out.	0·9% NaCl	5 ml	0·40	
Inn.	Cytosol fraction	1 ml	0·21	$\frac{1}{2·1}$
Out.	Distilled water	5 ml	0·44	

*0·25 M sucrose solution containing 1·0 μg corticosterone.

**Post-mitochondrial fraction, a mixture of adrenal microsomal (105,000 *g* pellet) and cytosol (105,000 *g* supernatant) fraction. Dialysis was carried out at 22–23°C for 24 h (see text for the details).

was carried out to ascertain a non-dialyzable corticosterone in the adrenal cell. A mixture of the microsomal and cytosol fraction was continuously dialyzed against saline for 6 days at 4°C until corticosterone could not be detected in the outer liquid. Every 24 or 48 h the outer liquid was replaced by fresh saline and

Table 3. Continuous dialysis of a mixture of adrenal microsomal and cytosol fraction

Inner liquid	Post-Mt fraction,* 1 ml		0·25 M sucrose containing 1·0 μg of Comp. B,** 1 ml	
Outer liquid	0·9% NaCl solution, 5 ml		0·9% NaCl solution, 5 ml	
	Corticosterone (μg) found in			
Dialysis No. (Duration)	Inner liquid	Outer liquid	Inner liquid	Outer liquid
1 (24 h)		1·247		0·715
2 (24 h)		0·383		0·216
3 (48 h)		0·095		0·066
4 (24 h)		0·121		0·048
5 (24 h)	0·362	0·000	0·000	0·000

*Post-mitochondrial fraction, a mixture of adrenal microsomal (105,000 *g* pellet) and cytosol (105,000 *g* supernatant) fraction.

**Corticosterone.

the dialysis was continued. The amounts of corticosterone in the outer liquids as well as that in the inner liquid poured out after the completion of dialysis were determined. The result obtained is shown in Table 3. It is noticed that a fairly large amount of corticosterone remained in the inner liquid even after corticosterone was not detected in the outer liquid.

DISCUSSION

In the present investigation evidence is presented that intracellular distribution of corticosterone in rat adrenal is influenced by hypophysectomy and ACTH administration. Evidence of specificity in the determination of corticosterone is based on a coincidence of fluorescence spectra of the determined substances in the adrenal subcellular fractions with that of the standard corticosterone. Since disruption and contamination of the subcellular particles could possibly occur to some extent during the course of homogenization and fractionation, the intracellular distributions of adrenal corticosterone described in the text probably do not give a complete information as to how much corticosterone exactly associated with the specific subcellular fractions. Changes in content and distribution of the intracellular corticosterone, however, are so distinct as to suggest influences of hypophysectomy and ACTH administration on the intracellular movement of adrenal corticosterone. It is usually thought that corticosterone formed in the adrenal mitochondria is moved into cytoplasm, then transported out of the adrenal cells by simple diffusion due to the differences between intracellular and extracellular corticosterone concentrations. It seems to be true on a result of experiments in which the time course-changes of adrenal and serum corticosterone are studied with rats given ACTH for stimulation of biosynthesis and secretion of the adrenal corticosterone. As shown in Fig. 5, an immediate increase of the adrenal

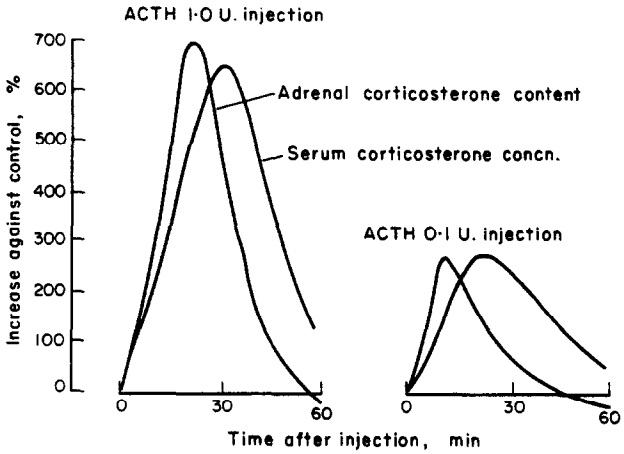

Fig. 5. Time course-changes in adrenal corticosterone content and in serum corticosterone concentration following a subcutaneous injection of ACTH.

corticosterone occurs following a subcutaneous injection of 1 unit of ACTH. A few min later an increase of the serum corticosterone follows. Maximal increase of corticosterone is achieved 20 min after the injection in the adrenal and 30 min after the injection in the serum. The increasing and decreasing pattern of the ad-

renal corticosterone after ACTH injection is quite similar to that of the serum corticosterone. Furthermore, the maximal increase in the serum corticosterone never exceeded that in the adrenal corticosterone. The result of this type of experiment, however, cannot permit the conclusion that secretion of adrenal corticosterone is merely due to a simple diffusion mechanism. A possible functional contribution of adrenal cell membrane to production and secretion of the adrenal corticosterone has been mentioned by Gomoll [3] and Wellen and Benraad [4]. Some electron microscopic studies have shown that fine structural changes in the adrenal intracellular organellae such as dilation of intramitochondrial vesicles followed by prolapse of the vesicles into the cytoplasm on chronic administration of ACTH to the rat [5], an opening of the mitochondrial membrane accompanied by a release of the mitochondrial contents into the cytoplasm by ACTH administration [6, 7], and a remarkable dilatation of endoplasmic reticula (ER) in the bovine adrenal on ACTH stimulation [8] would be related to the secretion mechanisms of the adrenocortical hormones. A more detailed study has indicated that a fullness of dilated tubular ER within the adrenal cell, a dilatation of anastomosing tubules of the agranular ER surrounding mitochondria and a frequent adhesion of limiting membrane of ER to external membrane of the mitochondria are typical electron microscopic features in fasciculata cells of the rat adrenal stimulated by ACTH [9]. These observations imply a possible contribution of the adrenal intracellular organellae to the adrenocortical hormone secretion. Our experiments give a biochemical meaning to these electron microscopic observations. As described in the text, corticosterone content of the microsomal fraction (which supposedly contains ER) does not usually fluctuate as much on hypophysectomy or on ACTH administration as the remarkable decrease of cytosol corticosterone on hypophysectomy and a marked increase on ACTH administration. It might indicate that the ER which provides the microsomal component is a reservoir of the adrenal corticosterone. The percentage of the microsomal corticosterone against the whole adrenal corticosterone in hypox rats is clearly greater than that in the intact rats, and it becomes smaller on ACTH administration. This fact will support the view that the adrenal ER component could be a reservoir of the adrenal corticosterone. From the data obtained and from the electron microscopic studies, one might be able to say that corticosterone biosynthesized in mitochondria would be moved in part directly from the mitochondria into cytoplasm but a significant amount of corticosterone in the mitochondria would be transported to cytoplasm or to the outside of the adrenal cell through the ER. Mixed procedures of intracellular transportation of the adrenal corticosterone such as a direct movement from mitochondria to cytoplasm, a movement from mitochondria to cytoplasm through ER and a movement from mitochondria to the outside of the cell through ER are most likely working in the mechanism of the adrenal corticosterone secretion, but it must be investigated further.

From the studies on binding of corticosteroids with the macromolecular substances [10–12], it seems possible to consider that the adrenal corticosterone exists within the adrenal cell not only in a free form but in a bound form which may play a significant role in the intracellular transportation of the hormone. The results of dialysis experiments have shown that a non-dialyzable form of corticosterone exists in the rat adrenal cytosol. Although experiments with labelled corticosterone will provide more precise information on the form of the adrenal intracellular corticosterone, data of the dialysis experiments shown in the text

suggest a possible existence of corticosterone bound to the macromolecules in the rat adrenal cytosol fraction. These experiments are, however, preliminary. Further investigations are now in progress.

REFERENCES

1. Guillemin R., Clayton G. W., Lipscomb H. S., and Smith J. D.: *J. lab. Clin. Med.* **53** (1957) 830.
2. Lowry O. H., Rosenbrough N. J., Farr A. L., and Randall R. J.: *J. biol. Chem.* **193** (1951) 265.
3. Gomoll A. W.: *J. Pharm. exp. Therap.* **156** (1967) 300.
4. Wellen J. J. and Benraad J.: *Biochim. biophys. Acta* **183** (1969) 110.
5. Sabatini D. D., DeRobertis E. D. P. and Bleichmer H. B.: *Endocrinology* **70** (1962) 390.
6. Nishikawa M., Murone I. and Sato T.: *Endocrinology* **72** (1963) 197.
7. Giacomelli F., Wiener J. and Spiro D.: *J. Cell Biol.* **26** (1965) 499.
8. Hechter O. and Halkerston I. D. K.: In *The Hormone*. Academic Press, New York, Vol. 5 (1964). p. 697.
9. Yoshimura F., Harumiya K., Watanabe M., Omoto T. and Sekiguchi T.: *Encocrinol. Japan.* **15** (1968) 145.
10. Morey K. S. and Litwack G.: *Biochim. biophys. Acta* **124** (1969) 260.
11. Fontana J. A., Walker M. D., Casper A. G. T., Meret S. and Henkin R. I.: *Endocrinology* **96** (1970) 1469.
12. Grosser B. I., Stevens W., Bruenger F. W. and Reed D. J.: *J. Neurochem.* **18** (1971) 1725.