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FREE PLASMA 11-HYDROXYCORTICOSTEROIDS IN PATIENTS WITH ADRENOCORTICAL DISORDERS

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Free acid-fluorescent corticosteroids (11-OHCS) in plasma of patients with adrenocortical disorder was studied by the method of De Moor. Differences in diurnal variations of 11-OHCS value were observed between healthy persons, patients with adenoma, and patients with hyperplasia of adrenals. Intravenous infusion of corticotropin was efficient for the estimation of the adrenocortical function. Dexamethasone test (a modification of ACTH suppression test by Liddle *et al.*) showed marked difference in the manner of response between obese persons and patients with some adrenocortical disorder. Although 11-OHCS decreased markedly after the administration of metopirone, it did not totally disappear. Those procedures mentioned above seemed to be convenient and useful for clinical evaluation of adrenocortical disorders.

INTRDUCTION

Various methods for determining the corticoids in plasma have been reported by many investigators. They can be classified in two groups: methods based on the Porter-Silber chromogen, and fluorimetries. The former, Nelson and Samuels' method[1] and its modifications, are difficult to apply clinically, because that (1) the sensitivity is rather low, (2) it needs much plasma, and (3) it takes considerable time. Fluorimetry has an advantage in determining very small amounts of plasma corticoids: Sweat's method[2] is capable to detect 0.05 μg of cortisol, whereas the minimum detectable quantity of cortisol by Nelson and Samuels' method is 0.1 μg. Silber *et al.*[3] (1958) reported a modification of Sweat's method by which corticosterone can be determined fairly specifically. Their method was applied by De Moor[4] (1960) for the study of the human plasma. The present author made a clinical study of free acid-fluorescent corticosteroids applying De Moor's method.

MATERIALS AND METHOD

1. Reagents and Apparatus

1) Petroleum ether: G. R. with a boiling point from 60 to 90°C.

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2) Methylene chloride: G. R. (Tokyo Kasei) was used without further purification. In this fluorimetry, redistillation of this solvent did not diminish the blank value.

3) 0.1 N NaOH.

4) Concentrated sulfuric acid: S.U. (Mitsubishi Kasei).

5) Ethanol: Free from peroxide and aldehyde (Katayama Kagaku) was used without further purification.

The total reagent blank amounts to 17% of the value obtained for 0.5 μg of cortisol.

6) KOTAKI UM-1 fluorophotometer with $5 \times 1 \times 1$ CM Microcell. The exciting light from a mercury lamp was filtered with a primary filter (maximum: 436 mμ), and the fluorescence light passed a secondary filter (>520 mμ).

2. Clinical Materials

Ten patients with Cushing's syndrome, 2 patients with Conn's syndrome, 1 patient with adrenocortical hypofunction due to a long term administration of triamcinolone, 1 patient with acromegaly, 1 patient with hypothalamus tumor, 2 adrenalectomized persons with advanced breast cancer, and others were selected for this investigation. The following points were studied in each case: 1) diurnal variation of 11-OHCS, 2) response to ACTH, 3) suppression caused by administration of dexamethasone, 4) response to metopirone, 5) others.

3. Determination of plasma corticosteroids

The procedure used in the present investigation was the same as that described by De Moor[4] (1960). Two ml of the plasma was washed for 30 seconds with 3 volumes of petroleum ether (6 ml). One and a half ml of the washed plasma could easily be recovered and diluted with water to final volume of 7.5 ml. This plasma solution was then extracted with 15 ml of methylene chloride by shaking twenty times the glass stoppered extraction tube gently. After centrifugation of mixture about 12 ml of methylene chloride extract was obtained using a syringe with a long needle. The methylene chloride extract was washed with 1 ml of 0.1 N NaOH for 15 seconds. The 10 ml of the extract was mixed thoroughly with 2 to 2.5 ml of reagent for fluorimetry by shaking for 15 seconds. The reagent contained 25% (v/v) of ethanol and 75% (v/v) of sulfuric acid. Then methylene chloride layer was removed and discarded by suction. Five minutes after reaction, the measurement was done, using KOTAKI UM-1 fluorophtometer. Reagent blanks and standards for fluorimetry were measured at the same time. The standard was 0.5 μg of cortisol dissolved in 2 ml of ethanol-water and blank was 2 ml of water.

RESULTS

1. Examination of standard curve

Fig. 1 shows the standard curve of cortisol-sulfuric acid fluorescence by this method. A straight line was obtained from 0.5 μg to 0. From the variance of the values, the minimum dose for the precise determination was shown to be about 0.02 μg. More than 2 ml of plasma may be necessary for the determination of 11-OHCS of which concentration is supposed to be low.

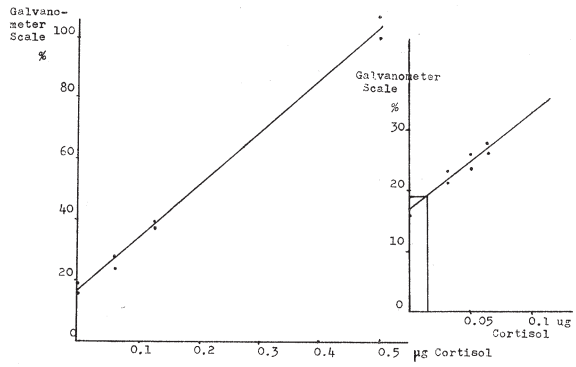


FIG. 1. Relations between concentration of cortisol and fluorescence in conc. H_2SO_4.

2. Recovery rate, Reproducibility, and influence of plasmavolume

Addition test was applied to check the recovery rate. When 0.05 μg or 0.1 μg of cortisol was added to 2 ml of plasma, the recovery after thorough process was 108 to 112 percent (Table 1).

Reproducibility of this procedure is also shown in Table 1. The first measurement was done in the morning, and the second in the afternoon on the same day. Good reproducibility was obtained.

Measurements were done with 2, 4 and 8 ml of the same plasma. The variations of 11-OHCS values were within the range of 10 per cent in all cases.

Investigations were made of the non-corticoidal fluorescence in plasma and of the influence of the fluorescence of solvent. An adrenalectomized patient with advanced breast cancer who had been discontinued the maintenance with cortisol 5 days since, showed non detectable level of free 11-OHCS. Another adrenalectomized patient who had been maintained with 5 mg per day of prednisolone also showed non detectable level of plasma 11-OHCS. A patient of Addison's disease showed a very low level of free plasma 11-OHCS (1.7 μg/100 ml). It can therefore be said that measurements were not interfered by the non-corticoidal fluorescence nor by the fluorescence of solvent.

TABLE 1

Recoveries of added cortisol

Samples	11-Hydroxy-corticoid μg/100 ml	% of Recovery
Original plasma	19.6	
Cortisol (0.05 μg) was added to the original plasma	25.2	112
Cortisol (0.1 μg) was added to the original plasma	31.0	103

Each values were determined by duplicate determinations.

Reproducibility of the values

Samples	1st Determination μg/100 ml	2nd Determination μg/100 ml
No. 1	3.4	3.6
No. 2	1.9	1.9
No. 3	2.6	2.7

The duplicate samples of the plasma were determined at different times of the same day.

The influence of the various volumes of the plasma

Volumes of the plasma	11-Hydroxy-corticoid
2 ml	19.6 μg/100 ml
4 ml	22.7 μg/100 ml
8 ml	22.0 μg/100 ml

TABLE 2. Plasma 11-OHCS in adrenalectomized patients and Addisonian disease

Adrenalectomized patient	at 6.00 not detectable
Adrenalectomized patient maintained with prednisolone	at 6.00 not detectable
Addisonian patient	at 14.00 1.7 μg/100 ml

3. Diurnal variation of free 11-OHCS in human plasma

Each 5 ml of blood drawn at 12, 18, 0 and 6 o'clock from healthy persons, patients with Cushing's syndrome due to adrenocortical hyperplasia or adrenocortical adenoma were submitted to the measurement of free 11-OHCS in plasma. The diurnal variations thus observed are shown in Table 3 and Figs. 2 and 3. The differences between these groups are shown in Table 4 on the basis of statistical calculations (Student's t). As can be seen in Table 3, the lowest value was obtained at 0.00 o'clock and the highest was at 6.00 in normal subjects. The range of variation was approximately 11 μg/100 ml, and its standard deviation (S) was the smallest at 0.00 o'clock and the largest at 6.00 o'clock. The difference was significant between the values at night and day (Table 4). In patients with adrenocortical hyperplasia, the level of free 11-

TABLE 3

Subjects			Time	Free plasma 11-OHCS μg/100 ml 12.00	18.00	0.00	6.00	
Normal								
N-1	24 yrs.	female		10.5	4.9	3.5	14.5	
N-2	24 yrs.	male		14.5	3.1	3.8	13.2	
N-3	13 yrs.	male					12.5	
							12.7	
N-4	30 yrs.	male		20.0	5.5	0	26 4	
N-5	38 yrs.	female		15.2	10.3	2.4	6.4	
N-6	34 yrs.	male		17.0	12.2	6.1	10.6	
N-7	27 yrs.	male		9.4	3.0	2.2	21.0	
N-8	30 yrs.	male		16.0	11.0	7.0	16.8	
Mean				14.7	7.2	3.6	14.9	
S				3.7	3.9	2.4	6.7	
Cushing's syndrome due to adrenocortical hyperplasia								
Hp-1	32 yrs.	female		21.0	17.0	10.9	11.0	Mean of 5 determs.
Hp-2	33 yrs.	female		15.0	19.0	21.5	19.7	Mean of 6 determs.
Hp-3	15 yrs.	female		21.0	13.2	19.7	23.7	Mean of 3 determs.
Hp-4	18 yrs.	female				18.0	11.0	
Hp-5	48 yrs.	female		19.2	11.5		23.0	
Hp-6	14 yrs.	female		15.1	11.5	21.0	25.6	
Mean				18.3	14.4	18.2	19.0	
S				2.6	3.5	4.3	6.5	
Cushing's syndrome due to adrenocortical adenoma								
Ad-1	27 yrs.	female		14.5	10.8	14.5	17.4	Mean of 5 determs.
Ad-2	18 yrs.	female		38.0	34.5	50.3	60.7	Mean of 3 determs.
Ad-3	34 yrs.	male		39.0	37.0	38.0	39.0	
Mean				30.5	27.4	34.3	39.0	
S				12.0	12.7	18.0	21.8	
Cushing's syndrome due to pituitary adenoma								
P-1	26 yrs.	female		17.6	15.4	26.0	17.6	
Conn's syndrome								
Co-1	33 yrs.	female		27.1	17.3	11.8	28.6	
Co-2	23 yrs.	female				19.8	27.7	
Acromegaly								
A-1	50 yrs.	female		15.9	18.4	16.0	28.6	
Hypothalamus lesion (terminal stage)								
HL-1	30 yrs.	male				68.0	50.0	
Advanced mammary cancer (terminal stage)								
AM-1	43 yrs.	female		19.7	21.4	19.0	15.5	
AM-2	52 yrs.	female		9.7	10.1	18.3	11.6	

TABLE 3. (*Continued*)

Subjects Normal		Time	Free plasma 11-OHCS μg/100 ml 12.00	18.00	0.00	6.00	
Prisoner expcsed in cold for long time							
Pr-1	18 yrs.	male	33.8	20.0	25.0	47.2	
Pr-2	18 yrs.	male	25.8	34.3	22.9	28.6	
Iatrogenic adrenal insufficiency							
Ii-1	43 yrs.	male	4.5	1.1	0.7	0.8	in disease
Ii-2					8.4	11.6	3 mos. after
Obese persons							
O-1	23 yrs.	female	18.8	17.5	13.6	26.5	
O-2	27 yrs.	female	33.3	20.1	23.2	41.4	
Mean			26.1	18.8	18.4	33.9	
S			10.1	2.6	6.6	5.8	
O-3			17.2	14.8	12.5	12.5	
O-4			41.6	16.8	28.7	21.1	
Mean			29.4	15.8	20.6	21.1	
S			17.4	1.0	11.4	12.3	

The diurnal variations of the whole subjects were shown. The signs (ex. Hp-1) were common to the tables and figures of this report.

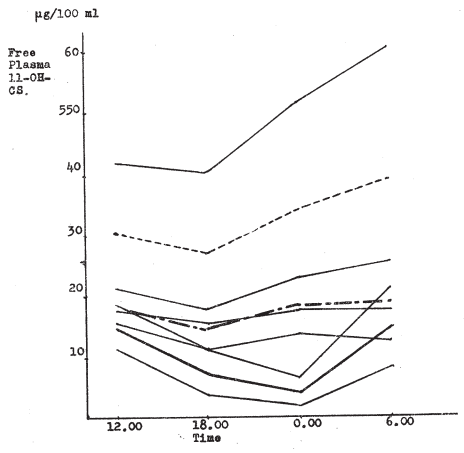


FIG. 2. Diurnal variations of normal persons (thick line), patients with adrenocortical hyperplasia (chain line) and patients with adrenocortical adenoma (dotted line) were shown. Standard deviations were also shown with thin line.

OHCS in plasma was higher than the normal value, with the exception of the value at 6.00. But the variation range was reduced (4.6 μg/100 ml). The mean lowest value of free plasma 11-OHCS was 14.4 μg/100 ml at 18.00. This value was much higher than that of normal. In patients with adrenocortical adenoma, the values of 11-OHCS were markedly high and widely distributed. The difference between normal and hyperplasia group was significant as indicated in Table 5. Their standard deviations were high with a range of 12.0 to 21.8 μg/100 ml. The patterns of diurnal variation in these two groups with

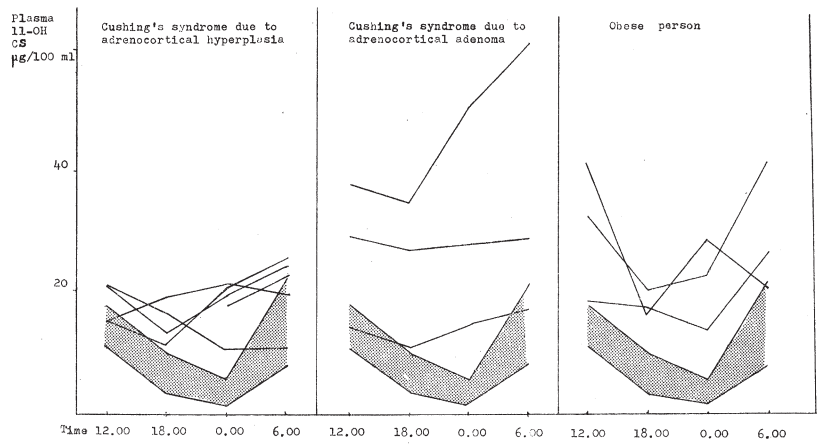


FIG. 3. Comparative illustration of diurnal variations of each groups of subjects. Dark area showed the normal (Mean±S.D.).

Cushing's syndrome became flat. It is of interest that the levels of 11-OHCS in obese persons were high and the variance was considerable. The concetrartions of plasma 11-OHCS at 0.00 and at 12.00 in obese persons were differed significantly from those of healthy persons, while no significant difference was observed between obese persons and patients with adrenocortical hyperplasia or adenoma. The pattern of diurnal variation and the level of free 11-OHCS in plasma of patient with Cushing's syndrome due to pituitary adenoma was the same as those of patient with adrenocortical hyperplasia. 11-OHCS values in plasma from 2 patients with Conn's syndrome were somewhat high. Two patients with breast cancer in the terminal stage had also high values and the patterns were unusual. A patient with tumor in hypothalamus had very high values and the pattern was inverse. Extremely low titer of plasma corticoids was obtained in a patient with adrenocortical hypofunction, due to triamcinolone administration. However the level increased as he recovered.

TABLE 4. Statistical examinations of the values at different times in normal person's by calculations of Student's t.

	Remarics according to Student'st
Difference between values at 12.00 and 18.00	highly significant
Difference between values at 18.00 and 0.00	not significant
Difference between values at 0.00 and 6.00	highly significant
Difference between values at 6.00 and 12.00	not significant

TABLE 5

Subjects	Time	Cushing's syndrome due to adreno-cortical adenoma		Cushing's syndrome due to adreno-cortical hyperplasia		Obesity-1		Obesity-2	
		F	t	F	t	F	t	F	t
Normal	12.00	##		−	+	##		##	
	18.00	##		−	##	−	##	−	+
	0.00	##		−	##	+	##		
	6.00	##		−	−	−	##	−	−
Cushing's syndrome (adrenal adenoma)	12.00			##		−	−	−	−
	18.00			##		−	−	−	−
	0.00			##		−	−	−	−
	6.00			+		−	−	−	−
Cushing's syndrome (adrenal hyperplasia)	12.00					+		+	
	18.00					−	−	−	−
	0.00					−	−	−	−
	6.00					−	+	−	−
Obesity-1	12.00							−	−
	18.00							−	−
	0.00							−	−
	6.00							−	−

Statistical investigations upon the differnce between each groups of patients were shown. ## means highly significant difference, and + means significant difference. − means "not significant difference".

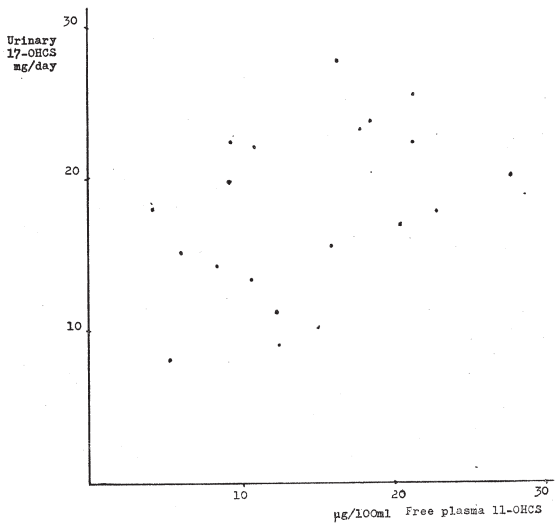


FIG. 4. Co-ordinate of free plasma 11-OHCS and Urnary 17-OHCS was shown. Correlation coefficient: 0.188.

4. Correlation of free 11-OHCS in plasma and urinary 17-OHCS excretion (Reddy and Jenkins' method)

Fig. 4 shows the correlation of 11-OHCS in plasma (at 0.00) and 17-OHCS in urine. It showed a tendency that the higher the level of 11-OHCS in plasma is, the larger the urinary 17-OHCS excretion is. However correlation coefficient was only 0.188, because the values were widely distributed.

5. Response to ACTH

The response to ACTH is shown in Tables 6 and 7.

a) Forty I.U. (2nd international unit) of ACTH (depot Scherring) was injected intramuscuarly at 6.00 or 8.00 (Tables 6-7, Figs. 5-7). The determination of 11-OHCS was done before and 1 to 12 hours after injection. Negative response was obtained in the patient with Cushing's syndrome due to adreno-

TABLE 6

Subjects	Free plasma 11-OHCS			Free plasma 11-OHCS		
	Basal	After i.m. inj. of ACTH (40 IU)		Basal	After i.v. inf. of ACTH (50 IU)	
		Max.	Time lag to Max. (hrs.)		Max.	Time lag to Max. (hrs.)
Normal subjects						
Normal	15.5	39.0	4.0			
Normal	12.7	19.9	4.5			
Normal				13.5	63.0	4.5
Cushing's syndrome due to adrenocortical hyperplasia						
Hp-1	12.5	30.8	3.0			
Hp-2	23.3	45.6	3.0	14.0	108.0	6.0
Hp-3	24.5	32.5	2.5			
Hp-5	23.0	54.0	2.5			
Hp-6	23.2	25.3	3.0			
Cushing's syndrome due topituitary adenoma						
P-1	17.6	40.8	3.0			
Cushing's syndrome due to adrenocortical adenoma						
Ad-1				25.0	63.2	6.0
Ad-2	92.0	44.5	10.0	40.0	44.0	12.0
Ad-3				42.0	75.0	12.0
Obese persons						
O-1	31.4	57.5	7.5	53.2	96.0	6.0
O-2	41.4	62.8	4.0			
O-4				29.7	91.0	9.0

Responses to ACTH were shown (i.m.: intramuscular, i.v.: intravenous, inj.: injection, inf.: infusion, Max.: maximum and hrs.: hours).

cortical adenoma as seen in Fig. 5, while the patients with Cushing's syndrome due to adrenocortical hyperplasia exhibited various values and could not be distinguished from obesity. It can be seen in Figs. 5 to 7, that one case with obesity exhibits a delay of response. The peak titer of responses in the patients with adrenocortical hyperplasia were obtained within 3 hours, the reason of these early responsiveness might be attributed to the hyperfunction of adrenals. As indicated in Fig. 11, the intramuscular administration of ACTH might not bring the condition of adrenals to full operation.

b) Fifty I.U. (2nd international unit) of ACTH (DAIICHI ORGANON) was infused continuously during the time period from 6.00 to 12.00. Before, during and after injection, the free 11-OHCS in plasma was determined (Tables 6–7). Fig. 9 represents the increasing curve of 11-OHCS during the period of observation. A patient with adrenocortical hyperplasia showed a high response.

TABLE 7

Subjects	Responses to ACTH administrations			
	After i.m. inj. of 40 IU ACTH		After i.v. inf. of 50 IU ACTH	
	Difference (Max.-Basal) μg/100 ml	Rate of rise %	Difference (Max.-Basal) μg/100 ml	Rate of rise %
Normal subjects				
Normal	23.5	153		
Normal	7.2	56		
Normal			49.5	366
Cushing's syndrome due to adrenocortical hyperplasia				
Hp-1	18.3	148		
Hp-2	22.3	96	94.0	665
Hp-3	8.0	32.6		
Hp-5	2.5	10.9		
Hp-6	2.1	8.4		
Cushing's syndrome due to pituitary adenoma				
P-1	23.2	132		
Cushing's syndrome due to adrenocortical adenoma				
Ad-1			38.2	152
Ad-2	−47.5	−52	4.0	10
Ad-3			33.0	78
Obese persons				
O-1	26.1	83	43.0	81
O-2	21.4	51		
O-3			61.3	206

Intramuscular and intravenous methods were compared with the amount and rate of responses produced,

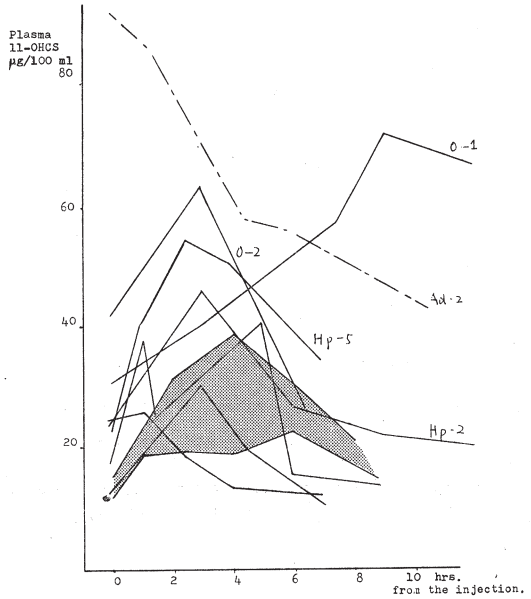


FIG. 5. Responses to i.m. injection of ACTH were shown. Forty IU of ACTH were injected at 6.00 or 8.00. Normal persons' were indicated with dark area, Cushing's patients' were with compact (hyperplasia of adrenal cortex) and chain (adenoma of adrenal cortex) lines. Obese persons' were shown with the compact lines.

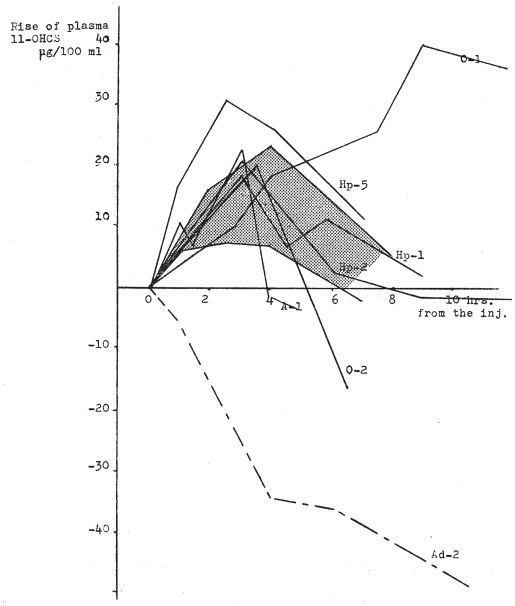


FIG. 6. Rise of the free plasma 11-OHCS (from base to max.) were shown. Normal persons were indicated with dark area, Cushing's syndrome were shown with compact (hyperplasia of adrenal cortex) chain (adenoma of adrenal cortex) lines. Responses to i.m. injections of ACTH 40 IU, were shown.

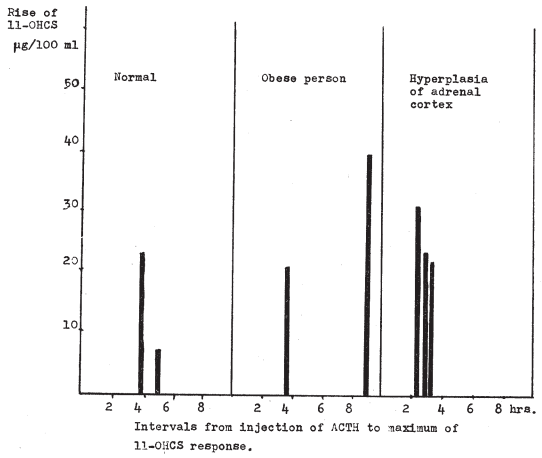


FIG. 7. Responses to intramuscularly administered 40 IU of ACTH were shown. The length of the pole indicated difference between base and maximum.

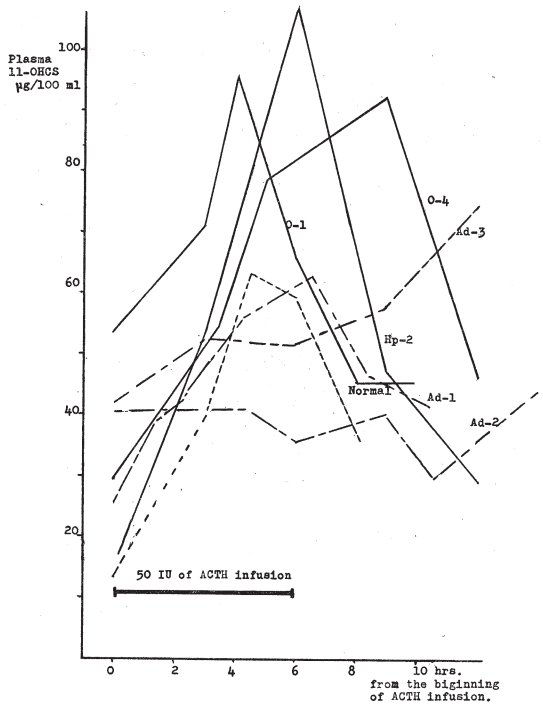


FIG. 8. Responses to 50 IU of ACTH infusion, were shown. Normal person: dotted line, Cushing's syndrome with adrenocortical hyperplasia; and obese persons: compact line, Cushing's syndrome due to adrenocortical adenoma: chain line.

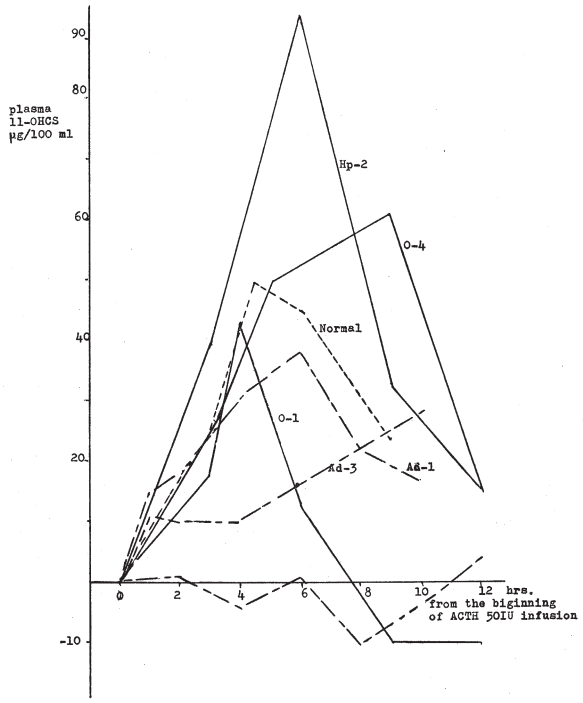


FIG. 9. Rises of plasma 11-OHCS were shown. Normal persons: dotted line, Cushing's syndrome due to adrenocortical hyperplasia and obese persons: compact lines. Cushing's syndrome due to adrenocortical adenoma: chain line.

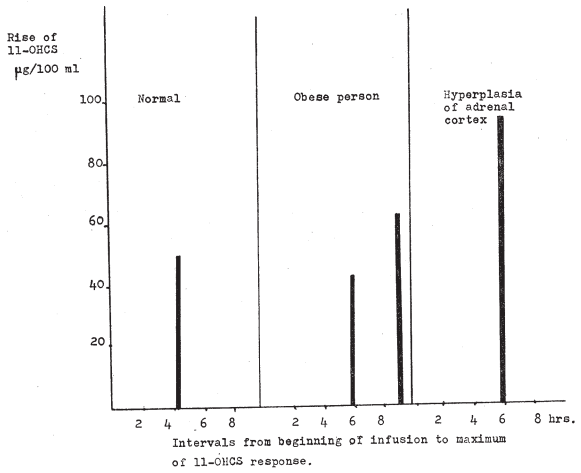


FIG. 10. Responses to infused 50 IU of ACTH. The ACTH was infused intravenously for 6 hrs. The length of the pole indicated difference between base and maximum.

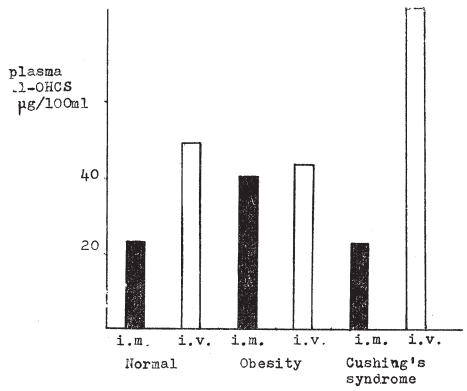


FIG. 11. Comparison of intra-muscular and intra-venous administration of ACTH.

No response was obtained in 2 patients with adrenocortical adenoma and one case showed an almost normal response. The obese subject who exhibited a delayed hyper-response to intramuscular injection of ACTH showed a normal response to this test, while a slightly higher response was observed in another obese subject as shown in Fig. 9. Fig. 10 shows the increase of 11-OHCS in plasma: the highest was that of hyperplasia, the median was that of obestiy and normal, and that of adenoma was the lowest. In an patient with Cushing's syndrome due to adrenocortical hyperplasia, the peak titer was seen at the end of infusion. If the infusion was prolonged, the peak titer might be higher than this. The increment of corticoids and the rate of increase were shown in Table 7. A

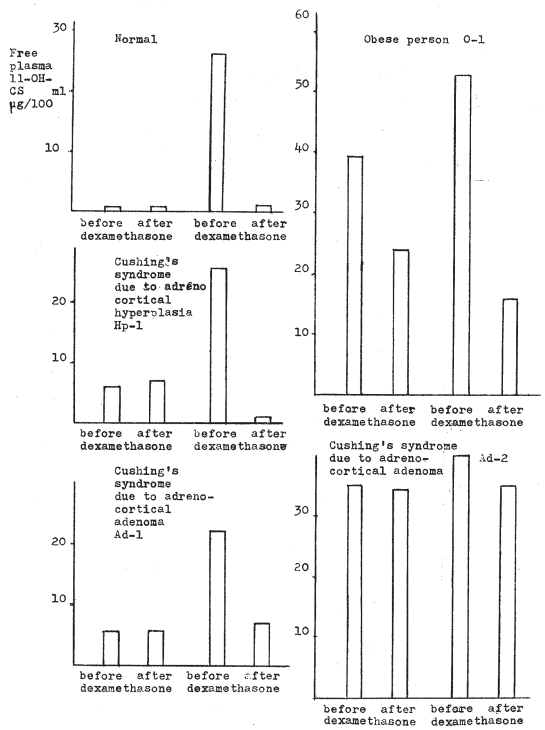


FIG. 12. Plasma 11-OHCS values at 0.00 and 6.00 during the suppression test.

TABLE 8

Subjects	Time	Basal	Free plasma 11-hydroxycorticoids μg/100 ml			
			1st day of dexametha-sone 2 mg per day	2nd day of dexametha-sone 2 mg per day	1st day of dexametha-sone 8 mg per day	2nd day of dexametha-sone 8 mg per day
Normal person						
Normal	0.00 6.00	n.d. 26.4	n.d. 18.0		n.d. 1.0	n.d. 5.0
Cushing's syndrome due to adrenocortical hyperplasia						
Hp-2	0.00 6.00	15.1 30.0		19.8 12.8		23.4 16.2
Hp-3	0.00 6.00	14.5 24.5				14.5 19.9
Hp-4	0.00 6.00	6.0 26.0		8.0 3.0		7.0 1.0
Hp-5	0.00 6.00	15.0 23.0		7.7		6.6
Hp-6	0.00 6.00	13.7 29.8		12.4 6.4		
Cushing's syndrome due to pituitary adenoma						
P-1	0.00 6.00	26.0 17.6			n.d.	n.d.
Cushing's syndrome due to adrenocortical adenoma						
Ad-1	0.00 6.00	5.4 22.1		10.8 11.6		5.4 6.5
Ad-2	0.00 6.00	35.0 40.0				34.4 34.7
Obese person						
O-1	0.00 6.00	39.5 53.0		34.4 18.9		23.4 16.2

Midnight value and morning value of plasma free 11-hydroxycorticoids before and during dexamethasone administration were shown. Two mg/day of dexamethasone were administered for 2 days and then 8 mg/day were administered for next 2 days.

patient with adrenocortical hyperplasia showed a response in normal range by intramuscular method, but by infusion method, the same patient showed a hyper-response (Table 7 Hp-2). Obese persons showed only normal or subnormal responses (Table 7). The result of infusion method was compared with that of intramuscular method (Fig. 11). It can be said that the former was more effective, because the responses were higher. Many discrepancies were seen in these two methods.

TABLE 9

		Basal	Free plasma 11-hydroxycorticoids μg/100 ml			
			1st day of 4.0 mg per day	2nd day of 4.0 mg per day	3rd day of 4.0 mg per day	4th day of 4.0 mg per day
Normal	0.00	3.5	2.0		2.0	
	6.00	14.5	6.5		4.0	
O-2	0.00	9.0		11.0		9.0
	6.00	19.0		17.0		27.0
Hp-1	0.00	10.8	9.4	18.3	15.0	16.5
	6.00	15.6	10.0		13.4	19.5
Hp-2	0.00	23.0	9.0	8.0	16.0	
	6.00	19.0	14.0	14.0	17.0	
Hp-6	0.00	4.1	14.5	19.5	19.5	16.5
	6.00	18.6	13.0	20.6	19.1	
Conn-1	0.00	11.8	14.9	10.6		11.2
	6.00	28.6	10.9	23.5		23.5

Free plasma 11-OHCS reduction by oral administrations of Metopirone 4.0 mg/day for 4 days were shown.

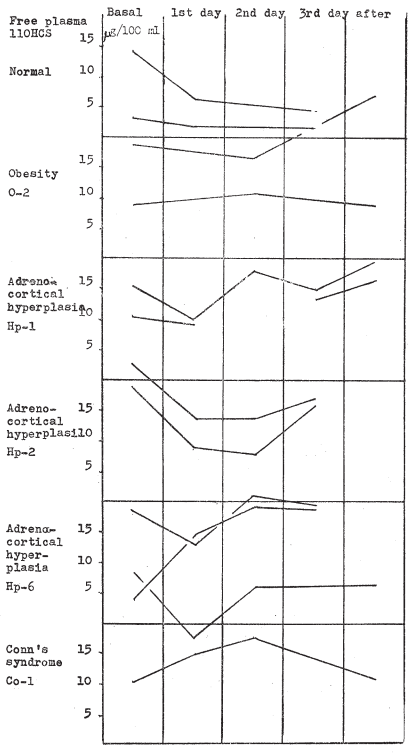


FIG. 13. Response to oral administration of metopirone (4.0 g/day).

6. Suppression test with the administration of dexamethasone (Table 8, Fig. 12)

Eleven cases were studied. Following the method by Liddle *et al.*[5] (1960), 2 mg of dexamethasone was administered on the 1st and the 2nd day, and 8 mg on the 3rd and 4th day. 11-OHCS in plasma was deternined at 0.00 and 6.00, before the administration, the 2nd and 4th day of administration. Marked suppression was observed in normal control. No marked suppression was seen at 0.00, but the value at 6.00 was suppressed in 5 patients with Cushing's syndrome due to adrenocortical hyperplasia, and 1 patient with adrenocortical adenoma. The latter also responded to intravenous ACTH administration (Fig. 9, Ad-1). But in another case of adrenocortical adenoma, no suppression was observed and the response to ACTH was also negative.

7. Metopirone test

a) Metopirone (Su-4885) test was performed by the administration of 4.0 g of metopirone orally for 3 days. In normal subjects, the level was lowered and the diurnal variations were reduced to practically zero (Table 9, Fig. 13). In two cases with adrenocortical hyperplasia, diurnal variations were decreased, but their levels did not alter so much. In one case with adrenocortical hyperplasia, the diurnal variation remained unchanged, but a fall of level was observed. In short, a suppression was observed in hyperplasia group on the 1st day, and a rise of level followed and higher level was maintained thereafter.

TABLE 10

Subjects	Basal		Free 11-OHCS in plasma μg/100 ml Response to metopirone infusion							
Time	0.00	6.00	8.00	10.00	11.00	12.00	13.00	14.00	16.00	Min.
Normal	3.8	13.2	8.3	5.7		4.2		2.6	8.8	2.5
Hp-5	2.1	14.6	7.2	9.5		3.7	9.5	6.2	3.4	3.7
Hp-6*	11.2	15.8	12.8	7.8	3.7	5.3	5.3		17.0	3.7
Hp-6	9.4	15.6	7.0	7.8	5.0	7.8	17.8			5.0
Ad-1	21.5	28.9	16.0	14.0		9.4	17.9			9.4

Three g of metopirone were infused. The minimum values were compared. The influence of 6 hours infusion (from 6.00 to 12.00) was shown. *; In this case 1 gm of metopirone was infused from 8.00 to 10.00.

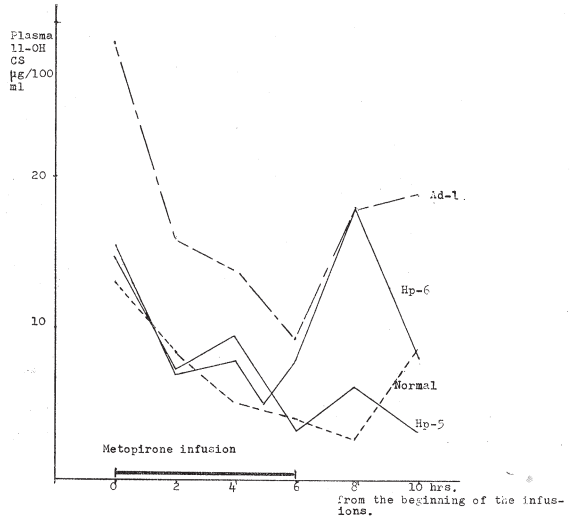


FIG. 14. Fall of free plasma 11-OHCS by infusion of metopirone was shown. To a normal person, 1.0 g was infused and the line was dotted. To Cushing's patients 3.0 g was infused. The Cushing's patients' with adrenocortical hyperplasia were shown with compact lines, and with adrenocortical adenoma was shown with chain line.

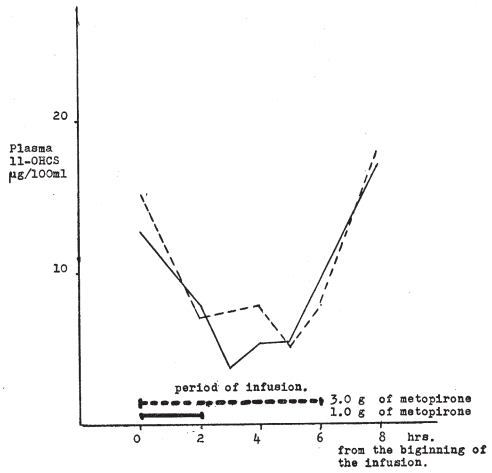


FIG. 15. Responses to metopirone infusion were shown. Influence of two metopirone dosages was examined.

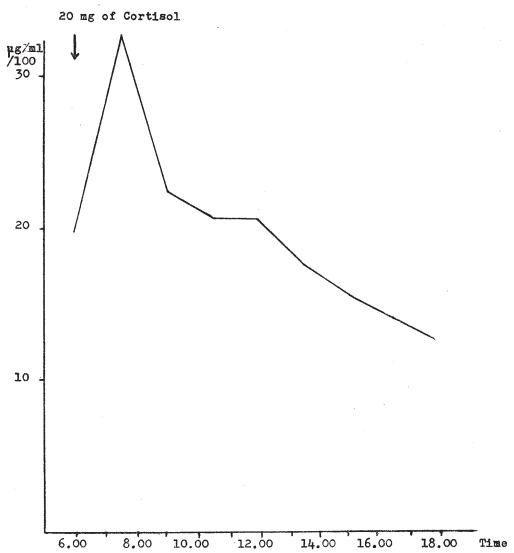


FIG. 16. Change of free plasma 11-OHCS concentration after administation of cortisol was shown. Twenty mg of cortisol was administered orally at 6.00.

A patient with Conn's syndrome also showed the same diagram as hyperplasia group.

b) Metopirone test by intravenous infusion

A dose from 2.5 to 3.0 g of metopirone was infused intravenously for 6 hours to 1 healthy person, 2 patients with adrenocortical hyperplasia and 1

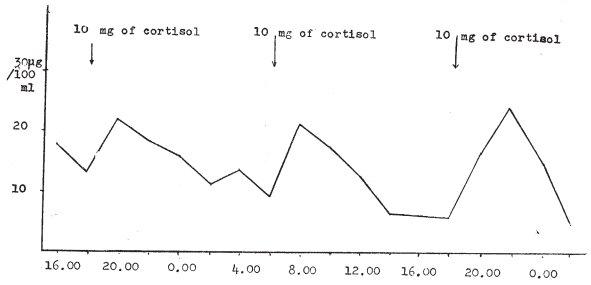
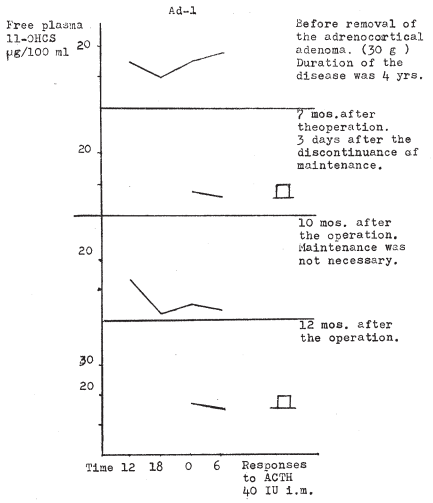


FIG. 17. Concentrations of free 11-OHCS were determined every 2 hrs. A 10 mg of cortisol was administered orally at 12 hrs. intervals.



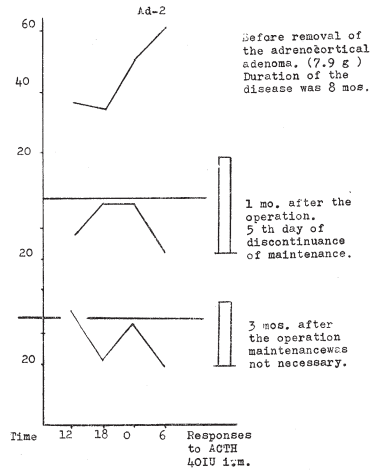


FIG. 18 FIG. 19

FIG. 18. Changes of the plasma 11-OHCS by recovery of the disease. (in case of Ad-1).

FIG. 19. Changes of the plasma 11-OHCS by recovery of the disease. (in case of Ad-2).

patient with adrenocortical adenoma. The minimum level of 11-OHCS did not reach zero in any of those cases. Among them, in the patient with adenoma, in whom a response to ACTH was observed, the fall of the level was the largest, but even his minimum value was rather high.

c) Influence of metopirone injection

Supression were observed by the intravenous infusion to the same subject either 1.0 g and 3.0 g of metopirone. As seen in Fig. 15, the injection of 1.0 g of metopirone may be enough to produce full inhibition, because the minimum value was the same as that of the dose of 3.0 g. Conclusively, it was difficult to distinguish the hyperplasia from adenoma of adrenals by this infusion

method.

8. Influence of abolition of cortisol maintenance in adrenalectomized subject (Fig. 16)

One tablet containing 20 mg of cortisol was given at 6.00. Free 11-OHCS in plasma was determined before and after the administration. About 3 hours after administration, the level of plasma 11-OHCS reached the maximum, then decreased gradually and it fell to zero, 5 days later. The patient became anxious at this time.

9. Influence of cortisol to the adrenalectomized patient

A tablet containing 10 mg of cortisol was administered at 6.00 and 18.00. The diagram of 11-OHCS level became biphasic (Fig. 17).

10. Observation in the patient from whom the adenoma of adrenal was removed

Patient 1 (Ad-1 in Table 2)

Fig. 18 shows the diurnal variations and responsiveness to ACTH measured at intervals. This patient required a long time to recover.

Patient 2 (Ad-2 in Table 2).

Fig. 19 shows the diurnal variations and responsiveness to ACTH. This patient regained the health in a short time.

DISCUSSION

De Moor omitted the chromatographic procedure in determining corticosteroids. His method is consisted of prewashing with petroleum ether, extracting with methylene chloride, then washing with aqueous sodium hydroxide. This method is simple and not time-cosuming, therefore this technique was thought to be suitable for a clinical use. But later, it was found that there was a trouble with noncorticoidal "plasma blank" fluorescence due to its simplified extraction prcedure. In 1961, Van der Vies *et al.*[9] attempted to separate cortisol from corticosterone by adding carbon tetrachloride partition to the method of De Moor. Braunsberg *et al.*[7] (1963) reported some improved method in which they used 8 ml of plasma to detect the free 11-OHCS. In order to avoid the contamination and decrease the blank fluorescence, Rudd *et al.*[8] (1963) added washing with carbontetrachloride. De Moor and Steeno[9] (1963) pointed out that the others' methods were not so excellent because they gave less specific fluorescence and higher reagent blank. However they reported that all the methods above mentioned brought well corresponded results (1963). Daly[10] omitted prewashing with petroleum ether and washing with aqueous sodium hydroxide in the De Moor's method, and reported that the reading on the fluorimetry at 5 minutes after mixing of reagents was the most specific and reliable. But the specificity of the method is considered to be slightly low (1964). The author's experience showed that De Moor's method fitted

for determination of plasma 11-OHCS in clinical investigation.

The fluorescence caused by other steroids was reported to be low, so that the administration of dexamethasone on the occasion of ACTH suppression test would produce only negligible amounts of fluorescence (De Moor[4] 1960, Mattingly[11] 1963). Neither the adrenalectomized oophorectomized cases nor the cases maintained with prednisolone after adrenalectomy showed detectable amount of plasma corticoids by this method. These findings indicate that the plasma fluorescence of non steroidal origin is thought to be as low as negligible in this method.

Concerning the diurnal variation, Doe *et al.*[12] reported that plasma 17-OHCS fluctuated the value from 13.6 μg/100 ml to 1.9 μg/100 ml, and its highest titer was obtained at 6.00 o'clock, the lowest at midnight. De Moor[4] reported that somewhat higher than these values. On the other hand, Peterson[13] (1957) reported that corticosterone in plasma fluctuated its value like cortisol, and the highest was 1.5 μg/100 ml at 8.00 and the lowest was 0.5 μg/100 ml at midnight. The sum of the values of 17-OHCS by Doe *et al.* and the values of corticosterone by Peterson *et al.* were in accord with the values of this study.

Vanishment of diurnal rhythm was observed in Cushing's syndrome by many investigators. Laidlaw *et al.*[14] studied the disappearance of diurnal variation in urinary 17-OHCS and Lindsay *et al.*[15], Doe *et al.*[12], in plasma corticoids. However Doe *et al.*[12], and Ekman *et al.*[16] reported that even in the cases with Cushing's syndrome, diurnal variation was observed, with irregular and high nocturnal values. In the present study, the difference of plasma 11-OHCS concentration at 0.00 between normal subjects and Cushing's patients was significant ($p<0.01$). It was difficult to distinguish obese persons from patients with Cushing's syndrome due to adrenocortical adenoma, because not only the Cushing's patients but some of the obese persons also showed high level and irregularity in plasma corticoids. Concerning this findings, Dankelman[17] showed that about half of the obese persons showed high level of plasma cortisol, and about half did not. Steigert[18] reported that about 60% of obesity showed adrenocortical hyperfunction. Mlynaryk[19] also reportrd the high secretion of cortisol, and Gogate[20] reported that it was difficult to distinguish obese from Cushing's patients. In this study, patients 0-1 and 0-2 showed high level of 11-OHCS. In cases with advanced breast cancer, high nocturnal level of plasma 11-OHCS was observed. This observation suggested the existance of adrenocortical hyperfunction in these patients, and this fact accords with the histological studies on the adrenals of these patients (Nishi[21]).

In this study, persons with higher levels of plasma 11-OHCS excreted much amount of urinary 17-OHCS but correlation coefficient among them was out of confidence interval, because the values were widely distributed (R: 0.188, $p>0.05$). The reason might be tnat the values of urinary 17-OHCS was discrepant with the real excretion of cortisol metabolites. These results were

observed by Ernest *et al.*[22]

Response to intramuscular administration of ACTH was uncertain, however the fact that the cases with adrenocortical hyperplasia showed quick reponses, might indicate hypersensitivity of adrenal cortex. A patient with adrenocortical hyperplasia showed a normal response to the intramuscular administration of ACTH. Much higher response was obtained by the intravenous administration of ACTH. This result indicates the superiority of intravenous method. Many studies were published concerning the ACTH test. Sandberg *et al.*[23] reported that 51 of 56 cases with adrenocortical hyperplasia showed high responses, but 8 of 11 cases with adrenal carcinema showed no responses to intravenous administration of ACTH. Soffer *et al.*[24] also reported that Cushing's patients due to adrenocortical hyperplasia or adenoma responded to ACTH. Christy *et al.*[25], Grumbach *et al.*[26] also reported that higher responsiveness was observed in Cushing's syndrome. Nabarro *et al.*[27] reported that one obese person, one healthy person, 15 of 23 cases of hirsute women and 7 of 11 cases of Cushing's syndrome showed overresponses. Beck *et al.*[28] failed to differentiate normal from Cushing's syndrome with intramuscular ACTH test.

The comparisons were made with the amount of increase or rate of increase, because the initial levels of plasma 11-OHCS varied with cases. Many overlappings were observed among the normal, obese, and Cushing's patients by intramuscular method, whereas intravenous method showed the responses separated clearly. By intramuscular method, the injection of ACTH must be done surely in the muscle. It is thought that unexpected results might be caused when the ACTH was injected intramuscularly, the reason was various concentrations of ACTH in plasma, even if the injections were done exactly into the muscle, the rate of dissolution to the tissue fluid and inactivation might be variable. From the reason mentioned above the intravenous method may be a surer method, and the comparison of the amount or rate of increase would be suitable.

The result of ACTH supression test by administration of dexamethasone were rather logical. Peterson *et al.*[29] reported that daily administration of 8 mg of dexamethasone produced very low secretion of cortisol. In this study, plasma 11-OHCS in normal and obese persons showed marked decrease both in midnight and morning, however patients with adrenocortical hyperplasia showed the decrease only in the morning. One patient with adrenocortical adenoma showed no suppression of plasma 11-OHCS. Another patient with adrenocortical adenoma showed the same type of suppression as hyperplasia. The results of this patient were unusual, but this patient responded to ACTH, so it might be thought that dexamethasone inhibited the adrenal cortex directly as explained by Fekte *et al.*[30] and Langeker *et al.*[31], or some degree of ACTH secretion persisted well.

Metopirone inhibits 11 β-hydroxylation in corticoid biosynthesis and in the present study, depressed the 11-OHCS values. A decrease occured at first, and

then an increase was observed. These findings coincided with the observation by Lazarus *et al.*[32] The hypocorticoidism produced by metopirone, stimulates the release of ACTH and accelerates the production of corticoids. As a whole, the decrease was not so marked. The reason why the range of the diurnal variation became so small was not clear, however it may be considered that maximum excretion of ACTH and maximum inhibition of 11-hydroxylation might result in the decrease of variation. This "negative feed back" mechanism also maintained the decrease of plasma level in slight degree. Metopirone infusion prodnced a marked decrease of 11-OHCS, but this brief duration of depression might not be expected to produced high level of ACTH, so only functional status of the adrenal cortex and not the responsibility of the pituitary adrenal axis would be observed. It was difficult to differentiate hyperplasia from adenoma by this infusion method. These results were also reported by Braunsberg *et al.*[7]

After total adrenalectomy, 20 to 30 mg of cortisol should be given orally every day. When it was administered every 12 hours, in divided dose, the diagram of plasma 11-OHCS value became biphasic and was not physiological. On the other hand, when it was given at one time, the curve became monophasic. Above all, when it was given at 6.00 the diagram became to be almost similar to the normal. Based on this result, the adrenalectomized with breast cancer or with Cushing's syndrome were maintained by single administration of cortisol in this series.

After the removal of adrenocortical adenoma, the recovery of atrophied adrenals was the first task. In some patients, recovery proceeds speedily but in others slowly. Graber *et al.*[33] (1965) reported that recovery of pituitary-adrenal axis after removal of adrenocortical adenoma needed more than 6 months from discontinuance of maintenance. But in this study, one took longer time and another took shorter time than that. After the recovery of the plasma corticoids level, the recovery to normal pattern of diurnal variation was delayed and more time was necessary. So the patients must be carefully observed until the rhythm of diurnal variation and response to ACTH are recovered.

CONCLUSION

Clinical application of plasma 11-OHCS determination with De Moor's method was studied. The results lead to following conclusions:

1) Diurnal variation of plasma 11-OHCS was a favorable indicator to know the pituitary adrenocortical function, althoughin Cushing's syndrome it was irregular.

2) The value of free plasma 11-OHCS at midnight was the most significant between those of healthy and Cushing's persons.

3) To estimate the function of adrenal cortex, ACTH should be infused intravenously. The rate of increase obtained by this method was a suitable index to diflerentiate healthy, obese and Cushing's persons.

4) To differentiate adrenocortical disorders, ACTH suppression test by the administration of dexamethasone was an adequate method in which the comparison of values at midnight was proved to be the most suitable.

5) The administration of metopirone decreased the range of diurnal variation. However clinical application of this test is thought to be questionable.

6) Oral administration of cortisol should be done at 6.00 o'clock in adreal-ectomized patients.

7) To estimate the recovery of adrenocortical function after removal of adrenocortical adenoma in Cushing's syndrome, the repeated determinations of diurnal varation in plasma 11-OHCS was useful.

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