

BIOSYNTHESIS OF VARIOUS STEROIDS IN VITRO BY ISOLATED ADRENAL CELLS IN PRIMARY ALDOSTERONISM, CUSHING'S SYNDROME, AND ADRENOGENITAL SYNDROME DUE TO ADRENOCORTICAL ADENOMA

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ABSTRACT

To a further understanding of the role of steroid hormones in adrenal disorders, we have prepared free cell system of adrenal cells, using adrenal tissues that had been removed by operation from (i) cases of Cushing's syndrome due to adrenocortical adenoma or adrenocortical hyperplasia, (ii) a case of primary aldosteronism, and (iii) a patient with virilizing adrenal tumor. Twelve important steroid hormones were measured, such as pregnenolone, cortisol and aldosterone, which were produced by these cells. Adrenal glands removed from the cases of advanced breast cancer were used for the measurement of steroids as a control. No organic lesions were confirmed in these adrenal glands. An investigation as to what characteristics the adrenal cells with various disorders show, had been carried out by examining the patterns of their steroid hormone production. The results were as follows: It was not the case that adenoma cells of each disorder produced just one of three types of steroid hormones, that is, glucocorticoids, mineralocorticoids, and androgens. In fact, it is interesting enough that each adenoma produced all steroid hormones. But, the quantitative pattern of each steroid hormone production differed characteristically from one disorder to the other. It was discovered that the symptom varies depending on the amount of the more predominantly produced hormone among these steroid hormones. Consequently, adrenal disorders can be classified into the three types before-mentioned. It seems that symptoms of these disorders were also modified by other steroid hormones being produced simultaneously. Cells of adenoma in the cases of Cushing's syndrome produced a fair amount of mineralocorticoids as well as cortisol. That is why hypokalemic alkalosis and hypertension in this disorders developed. In the case of Cushing's disease due to adrenal hyperplasia, steroid hormone production pattern was similar to that in the control. In the case of virilizing adrenal tumor, androgens were predominantly produced, with the increased production of cortisol. In fact, the latter finding was consisted with the patient's Cushingoid appearance. It was also discovered that the quantitative proportion of the various steroid hormones produced by isolated adrenal cells, varied depending on the cells which were placed on the different conditions. In the case of Cushing's syndrome due to adrenocortical adenoma, for example, the quantitative proportion of mineralocorticoids was higher where no ACTH was added to the medium, compared to that where ACTH was added. These results appear to correspond with the clinical findings of the above-mentioned disorders. And, it can be said that these data are convincing for explaining the clinical symptoms.

Keywords: Isolated Adrenal Cells. Cushing's Syndrome. Primary Aldosteronism. Virilizing Adrenal Tumor. Twelve Kinds of Steroids.

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INTRODUCTION

Among adrenal disorders due to overproduction of steroid hormones, three distinct disorders are known, as Cushing's syndrome, primary aldosteronism, and adrenogenital syndrome. They are said to be caused by the overproduction of different type of steroid hormones — cortisol, aldosterone, and androgens, respectively. At the same time, it has also been known that many cases that have these disorders are inclined to be accompanied by concentration abnormality of other steroid hormones in blood^{1,2,3)}. But, there have been no reports on their cause.

In recent years, we measured twelve kinds of plasma steroid hormones in patients with various types of Cushing's syndrome. And, we reported that there was abnormality in the plasma levels not only glucocorticoids but also mineralocorticoids, androgens, etc.⁴⁾ For example, in cases of Cushing's syndrome due to adrenocortical hyperplasia, the plasma level of androgens was high. In cases of adenoma, the plasma level of mineralocorticoids as well as glucocorticoids was high. It is possible that such high levels of these hormones cause clinical symptoms of either virilism or hypokalemic hypertension. And, this supports the view that the clinical findings of the above-mentioned patients were caused by such hormone abnormality. That is, we presume that these adrenal disorders showed abnormalities in all steps for steroid hormone synthesis.

The levels of steroid hormones in blood do not necessarily correspond directly with the productivity of adrenal glands. The reasons for this include, among others, the fact that metabolic clearance rate varies from one steroid hormone to the other. In order to find out the cause of abnormality in the level of these steroid hormones in blood, we prepared a free cell system, using various types of adrenal tissues that were removed by operation, and measured the productivity of various steroid hormones in these free cells.

MATERIALS AND METHODS

In this experiment, the following six patients were used: (i) 3 cases of Cushing's syndrome due to adrenocortical adenoma, accompanied by such symptoms as hypokalemia, hypertension (case 1, 2) and severe metabolic alkalosis (case 3), (ii) 1 case of Cushing's syndrome due to adrenocortical hyperplasia with slight hirsutism, (iii) 1 case of adrenogenital syndrome due to virilizing adrenal tumor with main symptom of hirsutism and low voice, and (iv) 1 case of primary aldosteronism due to adrenocortical adenoma. None of them received any spironolactone or potassium supplement before operation. They were all benign cases histologically and clinically. For preparing free cell system, only the adenoma tissue was used in the cases of adenoma. In the cases of adrenal hyperplasia and control, adrenal cells of total cortex were used.

(a) Free cell system and incubation. Immediately after removing the adrenal tissues by operation, free cell system were prepared, employing the method of Sayers *et al.*⁵⁾ Then, the cells were placed in a Krebs-Ringer bicarbonate buffer that contained 0.2% glucose and 0.5% bovine serum albumin, and incubated them for one hour, at 37°C, under 5% CO₂ and 95% O₂. The cells were divided into two groups; one group — ACTH(—) — to which nothing was added, and another group — ACTH(+)— to which porcine ACTH was added to be the final concentration of 500 pg/ml. The experiments were carried out in each group duplicate. In the case of adrenogenital syndrome, we also studied the response to hCG, which was added to the medium to be the final concentration of 0.1 μ U/ml.

(b) Extraction and assay. The steroid hormones were measured as follows: We extracted the sample with 15 volumes of dichloromethane. Subsequently, this dichloromethane was washed with 0.1N NaOH, 0.1N CH₃COOH, and redistilled water. After evaporating dichloromethane, we re-dissolved it in methanol; enclosed in an ampoule under N₂ gas spray; and preserved it at 4°C until hormone measurement.

The following 12 kinds of steroid hormones were measured: (i) precursor hormones: pregnenolone, 17 α -hydroxypregnenolone, progesterone, 17 α -hydroxyprogesterone, (ii) mineralocorticoids: 11-deoxycorticosterone, corticosterone, aldosterone, (iii) glucocorticoids: 11-deoxycortisol, cortisol, (iv) androgens: dehydroepiandrosterone, androstenedione, and testosterone. Steroid hormones were separated sufficiently with three sets of Sephadex LH 20 column chromatography using marker dyes. Corticosterone and cortisol were measured by means of competitive protein binding assay, and others by means of radioimmunoassay.

In the present study, each of 12 steroid hormones was measured at the same time with one assay system. The preservation period ranged from 1 to 8 months. The intra-assay coefficient of variation varied from one steroid hormone to the other. It ranged from 9.8 to 21%, with the average being 16.0%. Sensitivity as well as blank value of this method were altogether sufficient enough for this investigation. The details of this method have already been published⁶.

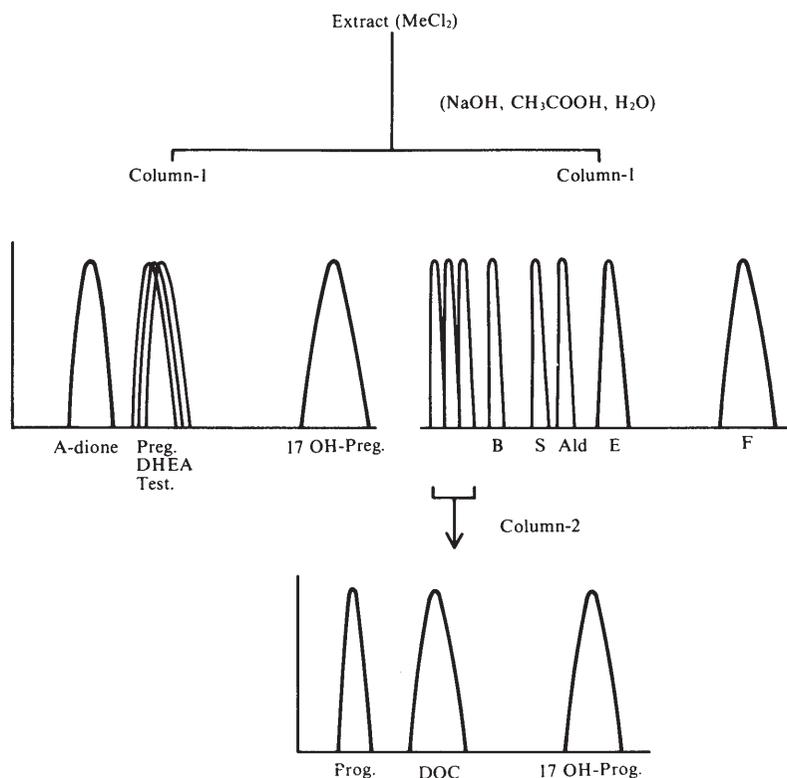


Fig. 1 Method for fractionation of the steroids; After extraction with MeCl₂ (dichloromethane) and washing, each steroid was separated by three sets of Sephadex LH 20 column chromatography.

Table 1. Amount of steroid hormones produced during one hour incubation of isolated adrenal cells ($\text{ng}/10^4$ cells/hr.). ACTH and hCG (human chorionic gonadotropin) were added to the medium to be the final concentration of 500 pg/ml and 0.1 $\mu\text{U}/\text{ml}$ respectively. Abbrev.: A.G.S.; adrenogenital syndrome, Prim. Ald; primary aldosteronism, Preg; pregnenolone, 17-Preg; 17 α -hydroxypregnenolone, Prog; progesterone, 17-Preg; 17 α -hydroxyprogesterone, DOC; 11-deoxycorticosterone, B; corticosterone, Ald; aldosterone, S; 11-deoxycortisol, F; cortisol, DHEA; dehydroepiandrosterone, A-dione; androstenedione, T; testosterone.

	ACTH	Preg	17-Preg	Prog	17- Prog	DOC	B	Ald	S	F	DHEA	A- dione	T	total	
Control	Case 1	(-)	7.1	0.80	2.2	0.61	2.1	18	0.30	7.3	92	3.4	12.4	2.3	148
		(+)	12.0	3.10	3.0	0.58	4.2	190	0.45	39.0	290	4.7	18.0	3.3	568
	Case 2	(-)	2.4	1.03	1.2	0.15	0.7	47	0.22	2.8	156	2.9	12.3	1.4	228
	(+)	6.5	1.40	1.3	0.75	1.2	75	0.28	18.8	163	4.3	18.0	1.3	291	
Case 3	(-)	12.5	1.30	4.7	1.20	2.1	53	0.36	16.2	104	6.7	51.0	3.3	256	
	(+)	21.0	5.20	7.6	0.49	4.6	50	0.32	18.0	97	9.1	38.0	2.9	254	
Case 1	(-)	4.1	0.66	1.7	0.22	1.7	7.2	0.19	6.5	88	1.1	3.7	1.9	117	
	(+)	9.1	0.73	2.1	0.39	1.7	8.8	0.15	6.5	60	1.3	10.0	1.9	103	
Adenoma	Case 2	(-)	7.6	0.90	2.2	0.44	3.8	16	0.81	10.6	86	1.2	22.9	1.8	154
	(+)	5.8	0.80	2.5	0.42	1.5	14	0.19	14.0	83	1.5	25.0	1.9	151	
Case 3	(-)	33.5	1.20	4.8	0.40	4.5	41	0.13	7.7	139	2.3	10.3	2.4	247	
	(+)	62.0	0.97	3.3	0.40	3.1	74	0.15	10.0	693	2.9	12.0	2.4	864	
Cushing	(-)	7.3	0.92	3.5	0.61	1.2	30	0.13	7.7	57	2.7	13.4	1.9	127	
	(+)	5.8	0.87	2.6	0.50	2.6	33	0.30	6.5	51	1.4	14.0	1.6	121	
Hyper- plasia	(-)	21.1	4.15	2.1	0.13	0.7	25	0.04	5.7	82	2.3	136	1.8	281	
	(+)	25.5	7.30	4.0	0.90	1.4	44	0.05	11.8	68	5.3	275	1.4	444	
A.G.S.	hCG	33.0	4.50	1.8	1.30	0.8	14	0.05	21.0	70	4.0	219	1.6	371	
Prim. Ald.	(-)	23.8	0.76	6.7	1.10	8.8	74	1.10	5.1	67	1.6	10.3	2.6	203	
	(+)	65.0	5.90	11.0	0.41	44.0	280	7.90	31.0	330	2.4	12.0	2.1	792	

RESULTS

The results of the present study are summarized in Table 1. Each value is expressed by $\text{ng}/10^4$ cells/hr. The 12 kinds of steroid hormones, which were measured, include all the important steroid hormones in the biosynthetic pathway. We regard their sum total provisionally as the total amount of steroid hormone produced, and indicate it at the right end. The total amount of steroid hormones, as can be seen in Table 1, varied markedly from case to case. That is why we have preferred to show each data as percent production of twelve steroids to absolute value in following figures.

In case of control, as expected, isolated adrenal cells produced all 12 kinds of steroid hormones. It was also the same in any case of adrenal disorders. Besides, in no disorder, did the total amount of production per unit cell show a big difference from that of control. However, what was produced predominantly was glucocorticoids in the case of Cushing's syndrome, mineralocorticoids in the case of primary aldosteronism, and androgens in the case of adrenogenital syndrome.

Fig. 2 shows the total amount of production, presented in Table 1. Response to the addition of ACTH in the final concentration of 500 pg/ml, was not constant, varying from one case to another. It is said that half of the cases of Cushing's syndrome due to adrenocortical adenoma lack response to ACTH. In our experiment, 1 case out of 3 responded, and increased by 250%. In case of adrenogenital syndrome also, increase by 58% was observed. The case of primary aldosteronism, which is said to be very sensitive to ACTH⁷⁾, increased by 290%. A case of the control and the case of Cushing's syndrome due to hyperplasia showed no change.

The percentage of production amount of each steroid hormone to the total amount of production, in each disorder, is shown in Fig. 3 and Fig. 4. The former shows the ACTH(-) group, and the latter the ACTH(+) group. When no ACTH was added, in the cases of Cushing's syndrome due to adrenal adenoma, 11-deoxycortisol and cortisol constituted from 59.4 to 80.6%. This shows that they are tumors that mainly produce glucocorticoids. But, there was a case whose production of mineralocorticoids was more than 18.4% and which showed a higher percentage of 11-deoxycorticosterone than the control. The percentage of androgens was small. In the case of adrenogenital syndrome, androgens, which mainly consisted of androstenedione, constituted 49.9%, more than about three times of the control. In the case of primary aldosteronism, we observed not only the increase of aldosterone, but also the increase of other mineralocorticoids in general, such as corticosterone, and they constituted 41.3%. The case of Cushing's syndrome due to adrenal hyperplasia showed a percentage similar to that of the control.

Addition of ACTH increased the total amount of production (Fig. 4). But it was not the case that the above-mentioned respective steroid was increased in a uniform way. Rather, it changed the production percentage of the steroid hormones relative to one another. In the control, the percentage of mineralocorticoids increased by the average of 59.4%. On the contrary, in the cases of Cushing's syndrome due to adrenal adenoma, the percentage of mineralocorticoids decreased by the average of 13.9%, increasing further the relative percentage of glucocorticoids production. In the case of adrenogenital syndrome, the percentage of androgens, which was predominant among the steroid hormone produced, further increased by 13.5%. At the same time, as in the control, the percentage of mineralocorticoids increased, while that of glucocorticoids decreased. In the case of primary aldosteronism, increase of the relative percentage of mineralocorticoids, observed in the control, did not occur, but all steroid hormones increased in a uniform way, remaining each

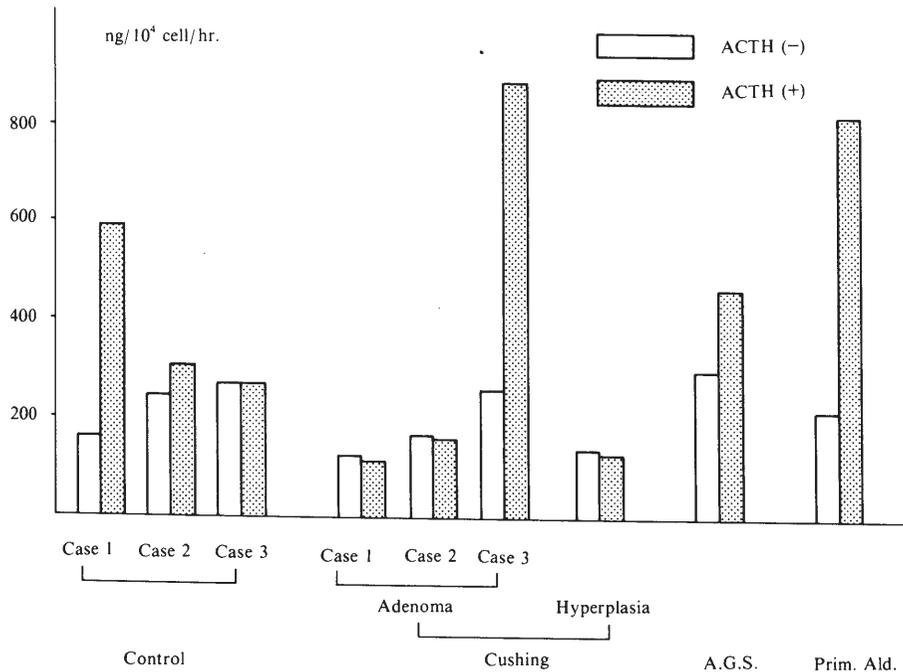


Fig. 2 Effect of ACTH on the production of steroids by isolated adrenal cells. Each bar shows a total amount of twelve steroids.

percent unchanged. In the case of Cushing's syndrome due to adrenal hyperplasia, the response to ACTH was approximately the same as that in the control.

In the case of adrenogenital syndrome, moreover, increase of androgen production was observed by the addition of hCG. The actual figures of these responses are shown in Fig. 5.

The amount of aldosterone produced was very small. But, it has marked vital effects on a living body. Therefore, the percentage of aldosterone to the total amount of production is shown in Fig. 6. The percentage of aldosterone production, observed in the tumors of the cases of Cushing's syndrome and adrenogenital syndrome, was similar to that in the control. The aldosterone percentage in both diseases decreased on the addition of ACTH, as in the control. In the case of primary aldosteronism, the percentage of aldosterone was naturally high, on the contrary, further increased when ACTH was added.

In many cases of Cushing's syndrome due to adrenal adenoma, plasma 11-deoxycorticosterone often shows a high level. Now, in order to estimate enzyme activity that is involved in the conversion of 11-deoxycorticosterone to corticosterone, we examined $(\text{corticosterone} + \text{aldosterone}) / (\text{11-deoxycorticosterone} + \text{corticosterone} + \text{aldosterone})$ as an index of 11 β -hydroxylase activity (Fig. 7). In each of the cases 1, 2, 3 of Cushing's syndrome, in whom plasma ACTH level is low, the index increased on the addition of ACTH. In the case of adrenogenital syndrome and control, in whom plasma ACTH level can be considered to be normal, the index showed no significant change. That is, in a state which lacks ACTH, the activity of 11 β -hydroxylase appears to be lowered.

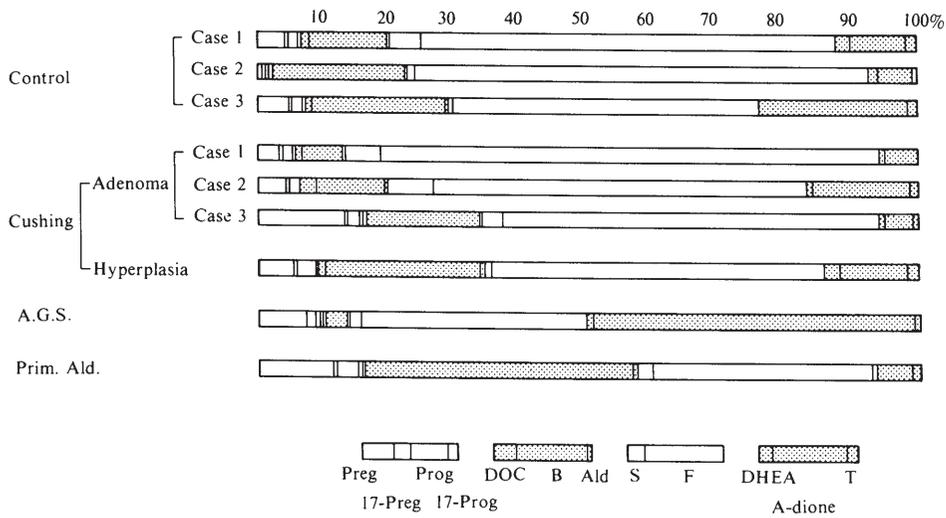


Fig. 3 Percent production of twelve steroids in ACTH(-) groups.

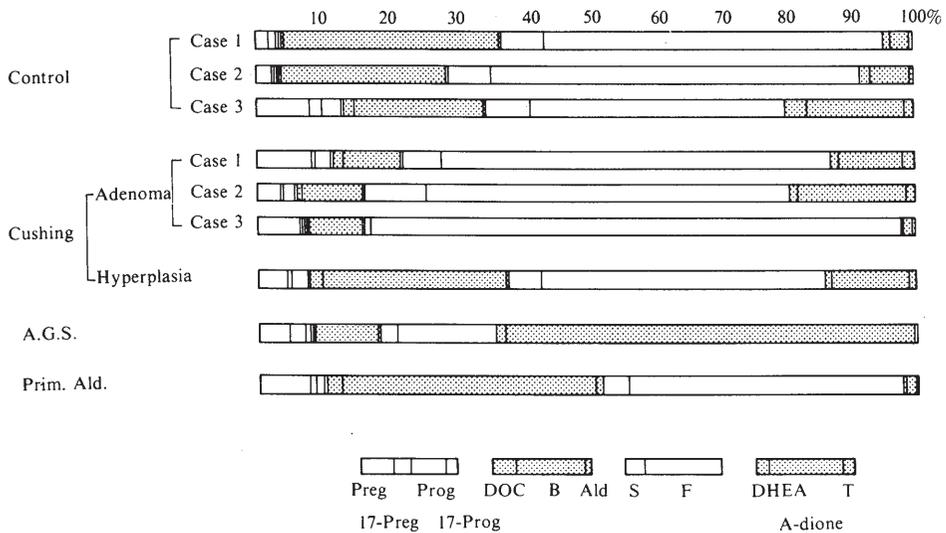


Fig. 4 Percent production of twelve steroids in ACTH(+) groups.

DISCUSSION

The proportion of 12 kinds of steroid hormones produced by isolated adrenal cells in case of control showed a considerable similarities to that found in plasma clinically, providing the possible availability of this method for the evaluation of the role of various steroid hormones on clinical findings in a variety of adrenal disorders.

Among the functional disorders of adrenal glands, Cushing's syndrome, primary aldosteronism, and adrenogenital syndrome are said to be mainly caused by

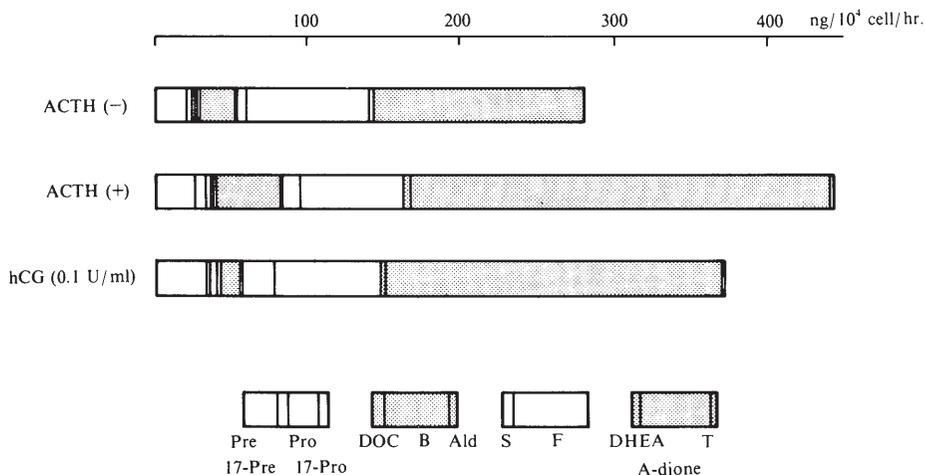


Fig. 5 Production of steroids in a case of A.G.S.

hypercortisolism, hyperaldosteronism, hyperandrogenism, respectively. However, we occasionally encounter some cases in whom these symptoms of three disorders are joining one another. In addition, as mentioned above, the pattern of plasma steroid hormone levels in cases of Cushing's syndrome varied depending on the different types of the disease⁴. Therefore, symptoms of such patients cannot be explained as an abnormality in the only one of these three types of hormone, *i.e.* cortisol, aldosterone, and androgen. It has been also known that adenoma in cases of primary aldosteronism produces cortisol *in vivo*⁹. That is to

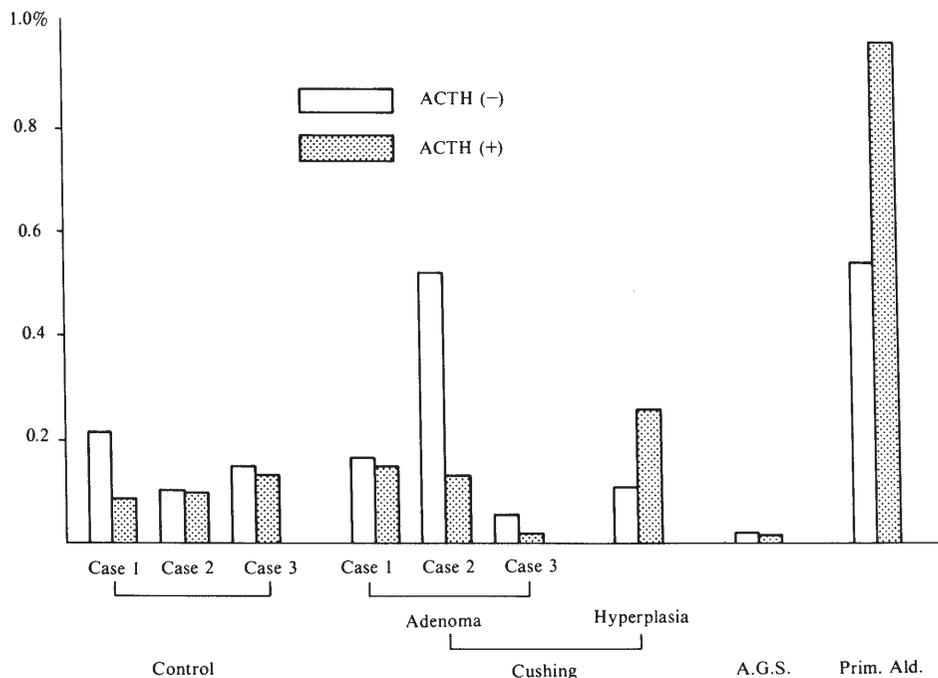


Fig. 6 Percentage of aldosterone to total steroid production.

say, pathological physiology of adrenal disorders remain still a matter of extreme complexity in many respects.

In order to elucidate these problems, it is important to measure various steroid hormones simultaneously in blood, which appear in different steroid hormone synthesizing pathways of adrenal glands. But, actually, there has been few reports in this respect. It is also important to evaluate the productivity of various steroid hormones individually *in vitro* in extirpated adrenal tissues. But, until present, to my knowledge, the only report on steroid hormone

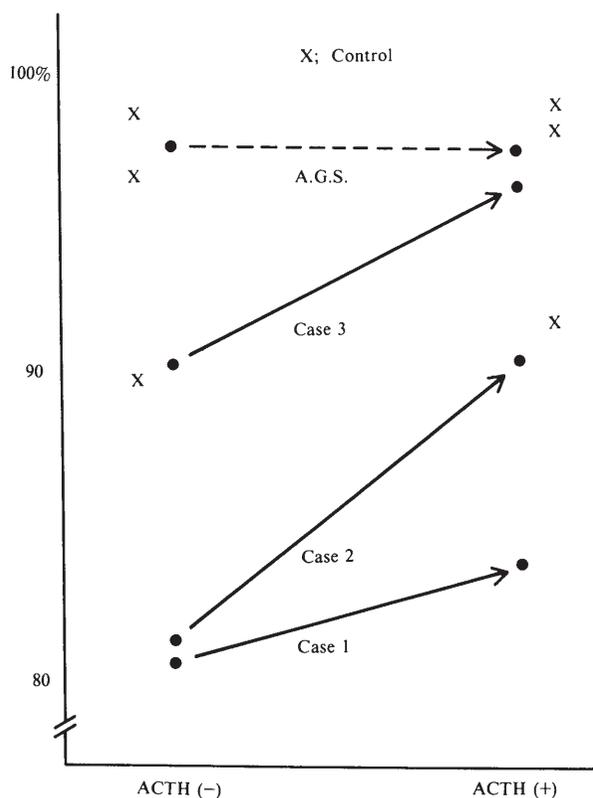


Fig. 7 11β -hydroxylase activity, estimated from (corticosterone + aldosterone)/(11-deoxycorticosterone + corticosterone + aldosterone), in Cushing's syndrome due to adrenocortical adenoma.

productivity covering all the synthesizing pathways is the one by Herkner *et al.*⁸⁾ He employed gas chromatography to measure the steroid hormones on a case of primary aldosteronism and a case of Cushing's syndrome due to adrenal hyperplasia. However, they did not correlate their results with clinical findings.

In our experiment, we removed adrenal tissues by operation from patients who mainly produced one of three types of steroid hormones, *i.e.* mineralocorticoids, glucocorticoids, and androgens, respectively. And, we prepared free cell systems. We then investigated what steroid hormones these cells produced and what influence they had on clinical findings. Since the present study concerns with steroid synthesizing activity of adrenal gland, results should have been expressed in molecular basis. However, because data expressed by $\text{mol.}/10^4$

cells/hr. did not show much difference from that by $\text{ng}/10^4$ cells/hr., the latter was provisionally used.

As Fig. 2 shows, the total amount of steroid hormone production per unit cell did not show any big difference between disorders and the control. And, small difference among cases was actually observed independently, irrespective of etiology of diseases. Although the reasons is not clear, difference in the cell damage during operative procedures may be a possible explanation for this.

The percentage of steroid hormone *in vitro* by free cell systems did not necessarily correspond with the percentage of steroid hormone by tumor *in vivo*. But, we presume that, if the tumor is big, abnormal steroid hormone production caused by the tumor will result in abnormality in the blood concentration of various steroid hormones. Particularly, in the cases of Cushing's syndrome due to adrenocortical adenoma, residual normal adrenal glands are atrophied drastically, and the gonads are in a hypofunctional state. The majority of steroid hormones in blood appear to have been produced by tumors.

Responses to the addition of ACTH by free cells were not uniform. On one case of control and one case of Cushing's syndrome due to adrenal hyperplasia, the amount of production did not increase. The biological half time of added ACTH in an incubation medium is said to range from 10 to 30 minutes. In order to find out steroid hormone productivity of cells in a physiological state, we set the ACTH concentration at 500 pg/ml at the beginning of incubation. In many experiments that have been reported, the ACTH concentration is higher than our experiment. This may be the reason why in our experiment, in contrast to many reported experiments, two cases did not respond to ACTH. The difference in the damages done to the cells during operation processes may possibly be another important reasons for this.

It is interesting that adenoma cells of each adrenal disorder produced all of the 12 kinds of steroid hormones. Moreover, they each produced mineralocorticoids, glucocorticoids, and androgens in a form not so much different from that of control. These results indicate that all steroid synthesizing enzymes are equipped, though in cases of congenital adrenal hyperplasia or adrenal cancer some defects of enzymes were found. (Unpublished data; estimated from steroid hormone patterns in peripheral blood.) That is, as long as they are benign adrenal disorders, their adenoma cells have all steroid hormone synthesizing systems. It appears that a given disorder turn out to be Cushing's syndrome, primary aldosteronism, or adrenogenital syndrome, depending on whether it mainly produce glucocorticoids, mineralocorticoids, or androgens, respectively. It must be added, however, that it is not known as yet whether there are differentiated particular cell groups, each of which, like normal adrenal glands, produce predominantly glucocorticoids, mineralocorticoids, and androgens, respectively.

The addition of ACTH increased the percentage of total mineralocorticoids in the control. This corresponds to the result *in vivo* where 11-deoxycorticosterone and corticosterone increased at a highest rate on the administration of ACTH⁶). On the contrary, in the adenoma of Cushing's syndrome, the addition of ACTH decreased the percentage of mineralocorticoids. In a state in which ACTH was added, which appears to be closer to a physiological state compared to a state not added, more glucocorticoids and less mineralocorticoids were produced. Accordingly, in a state in which ACTH was not added (clinically, as in the cases of Cushing's syndrome due to adrenocortical adenoma, where ACTH production was suppressed by the feedback mechanism), the percentage of mineralocorticoids was high. These results appear to explain the fact that cases of Cushing's syndrome due to adrenocortical adenoma often showed high level of 11-deoxycorticosterone,

corticosterone, and aldosterone in blood. In the case of primary aldosteronism, the addition of ACTH did not change the percentage of mineralocorticoids, but that of glucocorticoids increased. Kem *et al.*⁹⁾ reported, on the base of an experiment *in vivo*, that even with a minimal amount of ACTH, adenoma had produced cortisol. Therefore, in primary aldosteronism, adenoma cells appear to produce both mineralocorticoids and glucocorticoids.

It has been reported in many works that adrenal adenoma in Cushing's syndrome produce aldosterone^{1),7)} — both *in vivo* and *in vitro*. Generally, the decrease of the percentage of aldosterone due to addition of ACTH is known as intraadrenal effect¹⁰⁾. As Fig. 6 shows, in each of the three cases of Cushing's syndrome due to adrenocortical adenoma, the percentage of aldosterone decreased on the addition of ACTH, as in the control. This shows that the regulatory mechanism of the mineralocorticoid synthesizing systems in the adenoma was close to the normal. On the contrary, in the case of primary aldosteronism, the addition of ACTH increased the percentage of aldosterone. These results are compatible with a report that not only whole pathway of mineralocorticoids, peculiar to these disorders, but also the late step stage (conversion from corticosterone to aldosterone) was abnormally activated¹¹⁾.

It has been reported recently that 11β -hydroxylase activity depends on ACTH level¹²⁾. We obtained the same results in this experiment by using (corticosterone + aldosterone)/11-deoxycorticosterone + corticosterone + aldosterone) as the index of this enzyme activity. As Fig. 7 shows, in the control which is thought to have normal activity, the addition of ACTH did not cause any significant change in the index. But, in Cushing's syndrome due to adrenal adenoma (in which ACTH concentration in blood is low), the addition of ACTH increased this index in each of the three cases. In the case of adrenogenital syndrome, the response was the same as in the control.

On the contrary, when 11β -hydroxylase activity was assessed by (cortisol)/(11-deoxycortisol + cortisol), equivocal results were obtained. We presume that it may be resulted from the instability of 11-deoxycortisol in the medium used in the present experiment. (In contrast to 11-deoxycorticosterone, 11-deoxycortisol has degraded rapidly at a rate around 20 to 50% per hour in many cases. The degradation was confirmed by elution pattern in column chromatography of ³H-labelled 11-deoxycortisol, which was incubated for one hour in the same medium employed in this study: unpublished data).

From these results, it appears that in cases of Cushing's syndrome due to adrenal adenoma, more mineralocorticoids are produced as a consequence of low plasma ACTH level which was induced from suppression of ACTH by cortisol. Moreover, subsequent to lowered 11β -hydroxylase activity, the percentage of 11-deoxycorticosterone in all mineralocorticoids increase. This is perhaps one reason which explains high plasma 11-deoxycorticosterone level often observed in this disorders⁴⁾.

CONCLUSION

Adenoma of adrenal glands produce all steroid hormones. Depending on whether it predominantly produces glucocorticoids, mineralocorticoids, or androgens out of these steroid hormones, a given disorder turns out to be Cushing's syndrome, primary aldosteronism, or adrenogenital syndrome, respectively. In addition, the amount of other steroid hormones that are produced simultaneously, modifies the clinical findings of these disorders. At the same time, the change in the condition under which adenoma is placed, sometimes change the percentage of each steroid hormone produced by the adenoma. These various factors appear to influence one another, and manifest characteristic symptoms of

each disorder.

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