

Effects of ascorbic acid deficiency on adrenal mitochondrial hydroxylations in guinea pigs

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Abstract The effect of ascorbic acid deficiency on adrenal hydroxylation of cholesterol and deoxycorticosterone in guinea pigs was studied by using mitochondria and isolated cytochrome P-450 fractions. The effects obtained were compared with the effects of long-term treatment with ACTH. Advanced scurvy as well as treatment with ACTH resulted in an increase in the weight of the adrenals, the total amount of cytochrome P-450, the cholesterol side-chain cleavage activity, the cortisol level in plasma, and the excretion of unconjugated cortisol in urine. Total 11 β - and 18-hydroxylation of deoxycorticosterone were not stimulated or were stimulated only to a small extent. It is suggested that the major effects observed in advanced scurvy are due to ACTH, the level of which was significantly increased, most probably as a consequence of the stress. In animals kept on a scorbutogenic diet for 2–4 weeks or, with a small dose of ascorbate added, for several weeks, changes were observed that could not be fully explained as effects of ACTH on normal adrenals. Although the plasma levels of ACTH and cortisol were increased only to a small extent and excretion of unconjugated cortisol in urine was unaffected, there was a significant increase in the total capacity of adrenal mitochondria to hydroxylate exogenous cholesterol. It is concluded that the level of ascorbate in the adrenals might be of some importance for the capacity to convert cholesterol into pregnenolone. The normal feed-back regulation is, however, intact in moderate ascorbate deficiency and the plasma level of cortisol is kept within normal limits.

Supplementary key words cytochrome P-450 · mass fragmentation · cholesterol side-chain cleavage · 11 β - and 18-hydroxylation · scurvy

In spite of several efforts, the role of ascorbic acid in the biosynthesis of steroid hormones in the adrenals has not been completely established. Depletion of ascorbic acid in the adrenals is known to be associated with increased secretion of corticosteroids (1, 2) and the possibility has been suggested that this increased synthesis is related to an inhibitory effect of ascorbic acid on the hydroxylations involved in the biosynthesis of steroid hormones (2). Thus *in vitro* studies involving the addition of ascorbic acid to adrenal mitochondria have shown a direct inhibitory effect on side-chain cleavage of cholesterol and 11 β -hydroxylation

of deoxycorticosterone (3, 4). Sulimovici and Boyd (5) showed that ascorbic acid in physiological concentrations also inhibits cholesterol side-chain cleavage in ovaries and that this inhibition was a direct effect of the enzyme system, possibly cytochrome P-450.

ACTH is known to decrease the concentration of ascorbate in the adrenal gland (1). It has been speculated that this decrease of ascorbate decreases the inhibition of cholesterol side-chain cleavage activity and thus explains part of the stimulatory effect of ACTH (2). Evidence against such a mechanism has come from studies on ascorbate transport in adrenal glands of rats and guinea pigs (6). From these studies it was concluded that the effect of ACTH on concentration of ascorbic acid in the adrenal gland is mediated by the stimulatory effect of ACTH on steroidogenesis.

Further evidence against a primary role of ascorbic acid in steroidogenesis has been presented by Hodges and Hotston (7), who showed that in guinea pigs fed a scorbutogenic diet for 2 weeks, the adrenal concentration of ascorbic acid was less than 5% of the normal value and after 3 weeks on the diet, no ascorbic acid could be detected with the method used. After 3 weeks on the scorbutogenic diet, plasma cortisol concentration and excretion of 17-oxogenic steroids rose sharply. In spite of a low concentration of ascorbic acid in the adrenals, both histamine and corticotropin increased plasma corticosteroid concentration when injected during the second week, but failed to change the pre-existing high concentration of the steroids in the third week of ascorbic acid deficiency. It was suggested that the scurvy manifest in the third week is a severe stress and that this stress may increase the rate of synthesis of corticosteroids to such a level that it cannot be further stimulated by ACTH. It was not completely excluded, however, that the lack of effect of ACTH in the third week was due to lack of ascorbic acid in the gland at that time.

In previous studies, no attempts were made to determine the effect of scurvy on the different hydroxylations involved in adrenal biosynthesis of steroid

hormones. In the present work we studied cholesterol side-chain cleavage as well as 11β - and 18 -hydroxylase activity in mitochondrial fraction and partially purified cytochrome P-450 fractions from adrenals of scorbutic guinea pigs. The effect of scurvy on the different hydroxylations was compared with the effect of long-term treatment with ACTH. The effect of added ascorbate on the hydroxylations was also studied in the *in vitro* systems used. In view of a recent finding that long-term treatment with ACTH may affect side-chain cleavage of exogenous and endogenous cholesterol differently in the rat (8), a mass fragmentographic technique was used that allows determination of side-chain cleavage of both exogenous and endogenous cholesterol.

MATERIALS AND METHODS

Materials

[4 - ^{14}C]Cholesterol (specific radioactivity 59–60 $\mu\text{Ci}/\mu\text{mol}$) and [$1,2$ - $^3\text{H}_2$]deoxycorticosterone (sp act 10 $\mu\text{Ci}/\mu\text{mol}$) were purchased from Radiochemical Centre (Amersham, England). The purity of the labeled compounds was ascertained by thin-layer chromatography followed by radioscanning of the chromatoplates (cf. below). Prior to use, cholesterol was purified by chromatography on a column of aluminum oxide grade III (Woelm, Eschwege, Germany).

Corticosterone, deoxycorticosterone, and ACTH were obtained from Sigma Chemical Co. (St. Louis, MO). 18 -Hydroxydeoxycorticosterone was purchased from Steraloids (Pawling, NY). ACTH (Acton prolongatum) was obtained from Ferring (Malmö, Sweden).

Methods

Assay of cytochrome P-450, NADPH-cytochrome P-450 reductase, protein, cortisol, and ascorbate. Cytochrome P-450 was assayed from the absorbance of the carbon monoxide–cytochrome P-450 complex, after reduction with sodium dithionite, using an extinction coefficient of $91\text{ cm}^{-1}\text{ mM}^{-1}$ (9). NADPH-cytochrome P-450 reductase activity was assayed according to Masters, Williams, and Kamin (10) and expressed in units (one unit equals reduction of 1 nmol of cytochrome *c* per min). Protein was determined according to Lowry et al. (11). Urine was collected daily (from 8 AM to 8 AM) using metabolism cages in which feces and urine were separated. The urine was acidified and diluted to 100 ml prior to analysis. Plasma was collected when the animals were killed. Cortisol in plasma and urine were determined with a competitive protein-binding technique (12). It was previously

shown that this technique gave values very close to the values obtained with a specific mass fragmentographic method with a correlation coefficient of 0.96 between the two methods (13). ACTH was determined in plasma by radioimmunoassay using a commercial kit from Radiochemical Centre, Amersham, England. Ascorbate was determined in the 20% (w/v) adrenal homogenate according to Roe and Kuether (14).

Animal procedures and preparation of mitochondrial and submitochondrial fractions. Male colored guinea pigs were obtained from a local dealer. Their strain could not be identified. The initial body weight was about 200 g. If kept on an ascorbate-free diet, the animals died after about 6 weeks (15). One group of animals (control animals, Fig. 1 and Table 1) were kept on ordinary pellet diet and had free access to vegetables. Another group of animals (control animals for the ACTH experiment, Fig. 2) also received 0.3 ml of a 0.9% (w/v) sodium chloride solution subcutaneously at 8 AM. One group of animals (Fig. 1) was kept on an ascorbate-free diet (Astra-Ewos, Södertälje, Sweden) during the whole experiment. About every seventh day, one animal from this group and one from the control group were killed and the different adrenal hydroxylase activities were determined. Symptoms of ascorbate deficiency affecting fur and teeth developed after about 3 weeks. The last two guinea pigs developed severe scurvy. One of these two animals had hind limb paralysis.

Another group of animals (Table 1) was fed an ascorbate-free diet for about 3 weeks. When the animals stopped thriving, 0.5 mg of ascorbate in sucrose was given orally every second day for at least one week. With this treatment, the animals maintained their weights, but signs and symptoms of ascorbate deficiency were obvious, especially with respect to the condition of fur and teeth (15). The animals could be kept on this regimen for at least 4 months.

Another group of animals fed an ordinary pellet diet with free access to vegetables was treated with daily subcutaneous injections of ACTH, 2.5 I.U. in 0.3 ml of 0.9% (w/v) sodium chloride solution at 8 AM (Fig. 2 and Table 1). An animal from this group and one from the control group were killed on the 2nd, 5th, 6th, 7th, 8th, 10th, 16th, 22nd, and 25th day. All animals were killed at noon.

The mitochondrial fraction and insoluble cytochrome P-450 were prepared from a 20% (w/v) adrenal homogenate as described previously (16). A bovine NADPH-cytochrome P-450 reductase fraction (containing adrenodoxin and adrenodoxin reductase) was prepared from bovine adrenals (16). After centrifugation, the mitochondrial fraction was suspended in a small amount of distilled water (experiments with sub-

mitochondrial fractions) or diluted with Tris-Cl buffer, 0.1 M, pH 7.0, to a protein content of about 0.5 mg/ml (experiments with mitochondrial fraction). The insoluble cytochrome P-450 fraction was prepared after storage of the mitochondria for 12 hr at 4°C (16). The recovery of this cytochrome P-450 from mitochondria was always greater than 95% (16, 17).

Incubation conditions and assay of incubation products. In the standard incubation procedure, 80 µg of [1,2-³H₂]deoxycorticosterone (1.5×10^6 cpm) or 10 µg of [4-¹⁴C]cholesterol (1.5×10^6 cpm) was added to 0.5 and 1.0 ml, respectively, of the mitochondrial fraction fortified with 30 µmol of CaCl₂ and 3 µmol of NADPH in a total volume of 3 ml in 0.1 M Tris-Cl buffer, pH 7.0 (17). In incubations with reconstituted systems, the mitochondrial fraction was replaced by 0.1 nmol of the cytochrome P-450 fraction and 20 units of bovine NADPH-cytochrome P-450 reductase. In this case no CaCl₂ was added.

Prior to addition of substrate, the incubation mixture was preincubated for 5 min at 37°C. Incubations were performed for 10 min (cholesterol) or 20 min (deoxycorticosterone).

The incubations were terminated with methylene chloride (deoxycorticosterone) or chloroform-methanol 2:1 (v/v) (cholesterol). Extraction procedures, assay of labeled products, and analytical error have been described previously (8, 17). In order to assay conversion of unlabeled endogenous cholesterol, part of the chloroform extract of incubations with [4-¹⁴C]cholesterol was converted into trimethylsilyl ether and subjected to mass fragmentography as previously described (15). Estimations of endogenous cholesterol present in the mitochondrial preparations were performed by the same procedure after addition of a known amount of [4-¹⁴C]cholesterol of high specific radioactivity to 1 ml of the appropriate fraction prior to extraction.

In the incubations with mitochondrial fraction, it was ascertained that the conversion was linear with mitochondrial fraction and with time. The enzyme systems under study were saturated with respect to substrate. In the incubations with isolated cytochrome P-450, it was ascertained that the conversion was linear with amount of cytochrome P-450 and with time. The amount of NADPH-cytochrome P-450 reductase used, 20 units, was optimal under the conditions employed, and the system was saturated with substrate.

RESULTS

Fig. 1 summarizes the results of studies with animals fed a scorbutogenic diet only. The last animals in

the experiment developed severe scurvy (cf. Methods). The values given represent values from only one specific animal at each period of time.

The ascorbate content of the adrenals decreased to a low level during the first 3 weeks of treatment and then remained constant (Fig. 1A). The weight of the adrenals increased constantly during the study (Fig. 1B). Thus the last animals, which had been treated with the diet for about 6 weeks, had a ratio between weight of adrenals and body weight about twice higher than the control animals. The plasma cortisol level was similar in the treated and control animals for the first 4 weeks of treatment (Fig. 1C). After that period of time, the plasma level of cortisol rose sharply (7). The content of cytochrome P-450 in the adrenals rose after about 3 weeks on the scorbutogenic diet (Fig. 1D). The total capacity of the adrenals to cleave the side-chain of exogenous cholesterol increased after about 4 weeks of treatment with the scorbutogenic diet (Fig. 1E). This increase was due in part to an increase in the amount of cytochrome P-450 and in part to an increase in the specific catalytic activity of the cytochrome P-450. It was shown that cytochrome P-450 isolated from adrenals of guinea pigs fed the diet for more than 4 weeks had a higher capacity per nmol cytochrome P-450 to cleave the side-chain of exogenous cholesterol than had the corresponding fraction from control animals (Fig. 1G). The total capacity of the adrenals to cleave the side-chain of endogenous cholesterol could possibly be somewhat higher in the treated animals than in the control animals (Fig. 1F). It may be mentioned that the cholesterol content of the adrenal mitochondrial fraction gradually decreased during the treatment. Thus animals fed the diet for 6 weeks had a cholesterol content in the mitochondrial fraction of only about half that of the control animals.

The treatment was found to have only a slight effect on total 11β- and 18-hydroxylation of deoxycorticosterone (Fig. 1H). As is evident from Fig. 1D, this small increase was due to an increase in the total amount of cytochrome P-450.

The results given in Fig. 1 represent values from only one specific animal at each period of time; consequently it is not possible to make a statistical evaluation.

In **Table 1**, results are summarized from experiments with a homogenous group of animals kept on a scorbutogenic diet for 3 weeks and then given 0.5 mg of ascorbate every second day (15). There was a striking similarity between this group of animals and those fed the ascorbate-free diet for 2–4 weeks (Fig. 1) with respect to the different parameters studied. The treatment decreased the total amount of ascorbate in the adrenals by about 80%. The plasma level of cortisol

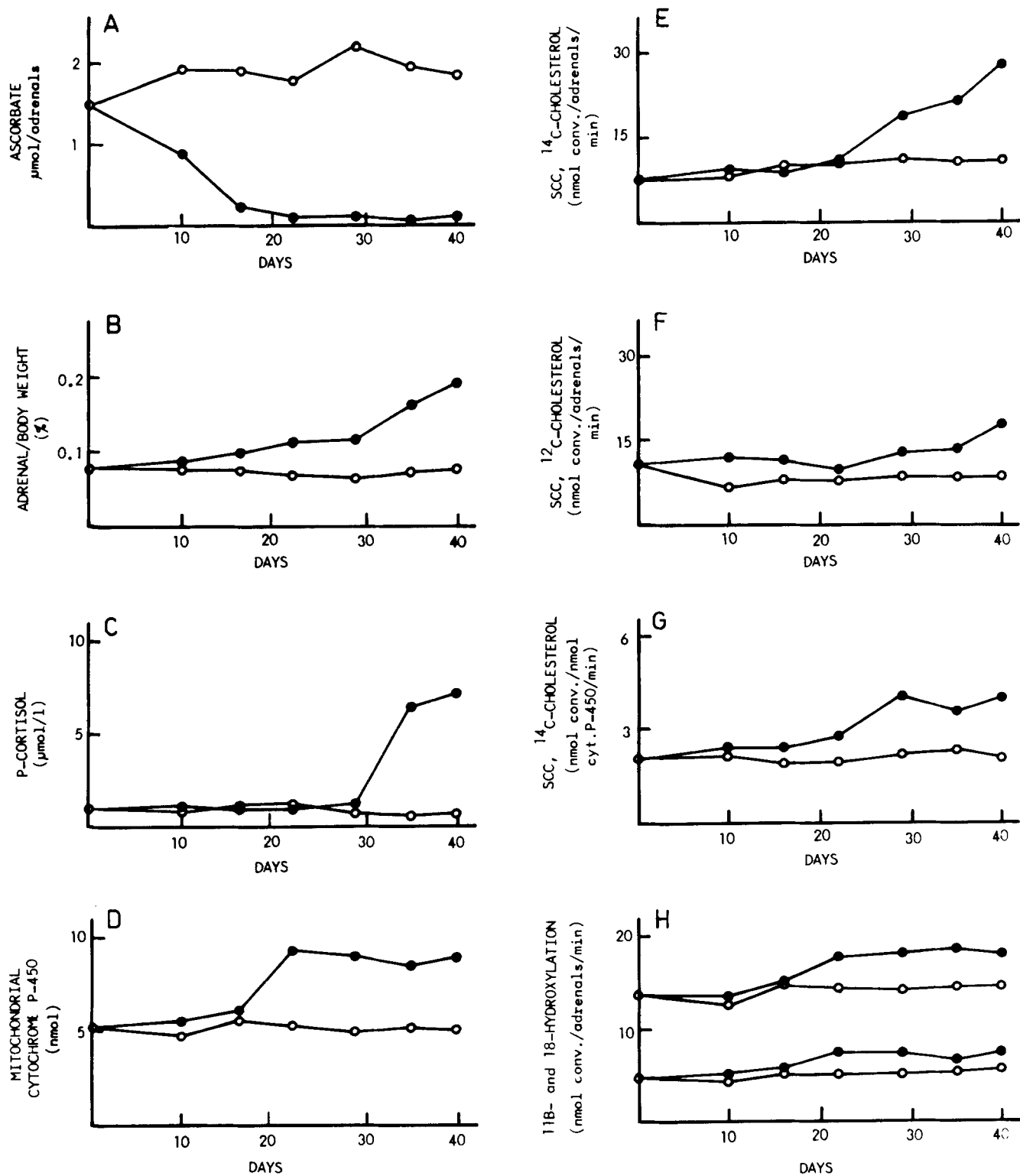


Fig. 1. Effect of a scorbutogenic diet on content of adrenal ascorbate (*A*), ratio between adrenal wet weight and body weight (*B*), plasma cortisol (*C*), mitochondrial cytochrome P-450 (*D*), mitochondrial capacity for side-chain cleavage of exogenous (*E*) and endogenous (*F*) cholesterol, side-chain cleavage of exogenous cholesterol in reconstituted systems (*G*), and 11 β - and 18-hydroxylation of deoxycorticosterone (*H*). Standard assay conditions were used. (●—●), Scorbutogenic diet; (○—○), control. In *H*, upper and lower parts correspond to 11 β -hydroxylation and 18-hydroxylation, respectively.

TABLE 1. Different effects of ACTH and of a scorbutogenic diet

	Control Animals	Scorbutic Animals	ACTH Treatment >10 days
	(n = 8)	(n = 6)	(n = 4)
Ascorbate, $\mu\text{mol}/\text{pair}$ of adrenals	1.19 ± 0.05^a	0.27 ± 0.03^d	1.62 ± 0.09^d
Ascorbate, $\mu\text{mol}/\text{g}$ wet weight	6.84 ± 0.15	0.87 ± 0.07^d	7.29 ± 1.06^d
Adrenal weight, mg	178 ± 4	257 ± 19^d	232 ± 23^d
Mitochondrial cytochrome P-450, nmol	3.8 ± 0.1	6.2 ± 0.1^d	8.0 ± 0.2^d
Side-chain cleavage of exogenous cholesterol, nmol converted/pair of adrenals/min in mitochondria	9.2 ± 0.4	22.6 ± 0.8^d	39.1 ± 2.0^d
Side-chain cleavage of exogenous cholesterol, nmol converted/nmol cytochrome P-450/min in mitochondria	2.4 ± 0.1	3.6 ± 0.2^d	4.9 ± 0.3^d
Side-chain cleavage of exogenous cholesterol, nmol converted/nmol cytochrome P-450/min in isolated cytochrome P-450	2.1 ± 0.3	2.5 ± 0.1^b	3.9 ± 0.0^d
Side-chain cleavage of endogenous cholesterol, nmol converted/pair of adrenals/min in mitochondria	11.8 ± 0.8	15.6 ± 1.4^c	26.3 ± 0.3^d
Side-chain cleavage of endogenous cholesterol, nmol converted/nmol cytochrome P-450/min in mitochondria	3.1 ± 0.2	2.5 ± 0.1^c	3.3 ± 0.1^b
11 β -Hydroxylation of deoxycorticosterone, nmol converted/pair of adrenals/min in mitochondria	10.9 ± 0.9	12.7 ± 0.8^b	12.4 ± 0.1^b
11 β -Hydroxylation of deoxycorticosterone, nmol converted/nmol cytochrome P-450/min in mitochondria	2.7 ± 0.5	2.3 ± 0.1^b	2.3 ± 0.3^b
18-Hydroxylation of deoxycorticosterone, nmol converted/pair of adrenals/min in mitochondria	3.3 ± 0.6	3.8 ± 0.8^b	4.5 ± 0.3^b
18-Hydroxylation of deoxycorticosterone, nmol converted/nmol cytochrome P-450/min in isolated cytochrome P-450	0.6 ± 0.2	0.5 ± 0.1^b	0.5 ± 0.1^b
Plasma cortisol, $\mu\text{mol}/\text{l}$	1.1 ± 0.1	1.6 ± 0.3^b	4.8 ± 0.3^d
Excretion of free cortisol, nmol/d	129 ± 16	110 ± 14^b	615 ± 52^d
Cholesterol content in mitochondria, $\mu\text{mol}/\text{pair}$ of adrenals	0.53 ± 0.01	0.47 ± 0.03^b	0.37 ± 0.02^d

^a $\bar{x} \pm \text{SEM}$.^b Not significant, Student's *t* test.^c $P < 0.05$.^d $P < 0.01$.

ACTH (2.5 I.U.) was administered daily subcutaneously in 0.9% sodium chloride solution. Guinea pigs given a scorbutogenic diet were fed 0.5 mg of ascorbate every other day for at least one week after an initial period on the ascorbate-deficient diet. Urine for cortisol determination was collected during the 24 hr prior to killing. For details see Methods.

and the excretion of free cortisol in urine were not significantly different from those of the control animals. Adrenal weight and mitochondrial cytochrome P-450 were, however, significantly increased in the treated animals as compared to the control animals. Total adrenal side-chain cleavage of exogenous cholesterol was increased more than twofold when using the mitochondrial fraction as source of enzyme. However, in a reconstituted system using isolated cytochrome P-450 fractions, there was no significant difference between treated animals and control animals with respect to catalytic activity per nmol cytochrome P-450. Side-chain cleavage of endogenous cholesterol was not significantly increased in the treated animals when using the mitochondrial fraction as source of enzyme.

11 β - and 18-Hydroxylation of deoxycorticosterone were not stimulated significantly by the treatment, regardless of whether crude mitochondrial fraction or isolated cytochrome P-450 was used as source of enzyme.

Effect of long-term treatment with ACTH

Fig. 2 summarizes results of studies with animals treated with ACTH. The values given represent values from only one animal at each period of time. The results were similar to those obtained with animals with advanced scurvy with respect to increase in adrenal weight, plasma level of cortisol, total amount of cytochrome P-450, and total capacity to cleave the side-chain of exogenous and endogenous cholesterol. The capacity to cleave the side-chain of exogenous cholesterol was increased in all animals treated for more than 2 days with ACTH. The increase was due both to increased amount of cytochrome P-450 and to increased specific catalytic activity of cytochrome P-450. It was shown that cytochrome P-450 isolated from the treated animals had a higher capacity to cleave the side-chain of exogenous cholesterol than did the corresponding fraction from control animals. The cholesterol content of the adrenal mitochondria decreased during the treatment and reached a value about half of that of the control animals.

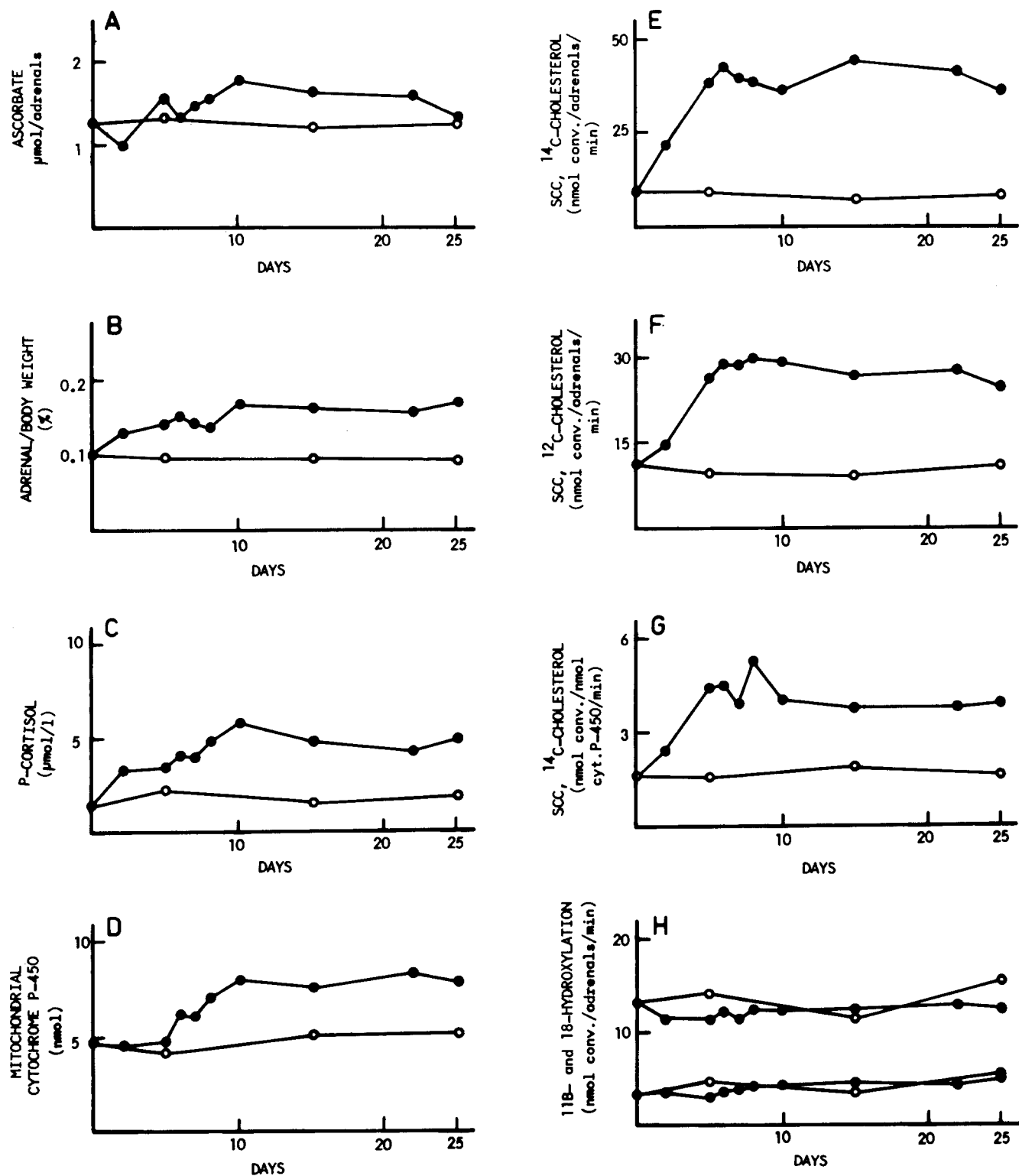


Fig. 2. Effect of treatment with ACTH on content of adrenal ascorbate (A), ratio between adrenal wet weight and body weight (B), plasma cortisol (C), mitochondrial cytochrome P-450 (D), mitochondrial capacity for side-chain cleavage of exogenous (E) and endogenous (F) cholesterol, side-chain cleavage of exogenous cholesterol in reconstituted systems (G), and 11 β - and 18-hydroxylation of deoxycorticosterone (H). Standard assay conditions were used. (●—●), Treatment with ACTH; (○—○), treatment with sodium chloride. In H, upper and lower parts correspond to 11 β -hydroxylation and 18-hydroxylation, respectively.

The total amount of ascorbate in the adrenals was found to increase slightly during the period of day 10 to day 20. The concentration of ascorbate, however, was slightly decreased, since the weight of the adrenals increased more than the total amount of ascorbate.

ACTH treatment was found to have little or no effect on 11 β - and 18-hydroxylation of deoxycorticosterone, regardless of whether crude mitochondrial fraction or isolated cytochrome P-450 was used as source of enzyme. When total capacity for conversion was calculated, a slight increase was found (Fig. 1H).

The results obtained with the guinea pigs treated with ACTH for more than 10 days were all similar. For reasons of comparison, these results have also been included in Table 1.

Determination of ACTH in blood of the different groups of animals

Table 2 summarizes results of determinations of ACTH in blood of the three different groups of animals. In accordance with previous studies (18) great individual variations were found within each group. The variations were smallest in the group of animals treated with the scorbutogenic diet for 7 weeks, and the ACTH level of this group was significantly higher than that of the control group. The concentration of ACTH in the animals fed the scorbutogenic diet together with a small dose of ascorbate was not significantly different from the corresponding concentration in the control group.

Inhibition of cholesterol side-chain cleavage activity in vitro by ascorbate

Fig. 3 shows the effect of adding ascorbate to an incubation of the mitochondrial fraction from a guinea pig (control animal) with [4-¹⁴C]cholesterol. In accordance with the work by Shimizu (3), a small amount of

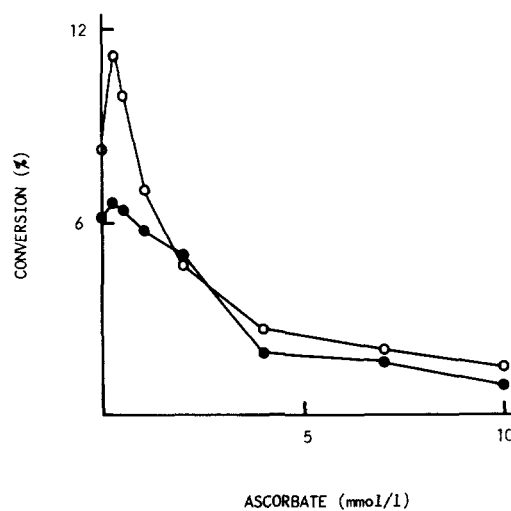


Fig. 3. Effect of added ascorbate on adrenal mitochondrial side-chain cleavage of cholesterol. Standard assay conditions were used. (○ — ○), [¹²C]cholesterol; (● — ●), [¹⁴C]cholesterol.

added ascorbate, corresponding to 0.1–0.5 mmol/l, slightly stimulated side-chain cleavage activity of both exogenous and endogenous cholesterol. With a higher concentration of added ascorbate, there was an inhibition of side-chain cleavage of both exogenous and endogenous cholesterol. Results similar to those shown in Fig. 3 were obtained when using isolated cytochrome P-450 as source of enzyme.

Parallel experiments were performed with adrenal mitochondria from control animals and from animals fed the scorbutogenic diet together with 0.5 mg of ascorbate every second day. In all these experiments, the inhibitory effect of a constant amount of added ascorbate (corresponding to 1.7 mmol/l) was higher with mitochondria from animals fed the scorbutogenic diet than with those from control animals. The inhibition of side-chain cleavage of exogenous and endogenous cholesterol was 26.3 ± 2.2% and 22.5 ± 2.5%, respectively, when using the preparations from control animals (means ± SEM from six animals) and 49.0 ± 4.9% and 52.0 ± 3.5%, respectively, when using the preparations from the animals fed the scorbutogenic diet. The differences were statistically significant in the case of side-chain cleavage of both exogenous and endogenous cholesterol ($P < 0.005$, Student's t test).

DISCUSSION

Effects of advanced scurvy

In consonance with the work by Hodges and Hotston (7), the present work shows that there are striking

TABLE 2. ACTH levels in blood of control guinea pigs and guinea pigs treated with the scorbutogenic diet

Animals	ACTH Concentration ng/l	P
Controls	161 ± 95	
Guinea pigs fed scorbutogenic diet for 7 weeks	355 ± 44	<0.01
Guinea pigs fed scorbutogenic diet plus 0.5 mg ascorbate every second day	311 ± 128	>0.05

The values given are the mean ± SEM from five animals in each group.

similarities between the effects of scurvy and long-term treatment with ACTH. This similarity was most pronounced in the case of advanced scurvy, which develops after the guinea pigs are fed the scorbutogenic diet more than 4 weeks. In these animals, which were found to have a significantly increased plasma level of ACTH, plasma cortisol was increased three to four times. This increase was paralleled with about a 100% increase both in content of adrenal cytochrome P-450 and in cholesterol side-chain cleavage activity per nmol of isolated cytochrome P-450. Almost identical changes were observed in the ACTH-treated animals. In a previous study in this laboratory, long-term treatment of rats with ACTH was studied (8). Also in that investigation, there was a significant increase in cholesterol side-chain cleavage activity per nmol cytochrome P-450. In contrast to the present study, and possibly due to species differences, there was little or no increase in adrenal content of mitochondrial cytochrome P-450. Furthermore, in that study, side-chain cleavage of exogenous cholesterol was affected more than side-chain cleavage of endogenous cholesterol. The latter finding might be due to differences in the adrenal content of cholesterol. Thus a high cholesterol side-chain cleavage activity *in vivo* decreases the amount of endogenous cholesterol available for the assay system *in vitro*. Such a decrease might lead to a relatively low formation of pregnenolone from endogenous cholesterol in spite of a high enzyme activity.

Effects of moderate scurvy

The effects observed in guinea pigs kept on a scorbutogenic diet for 2–4 weeks, or kept on a scorbutogenic diet with a small dose of ascorbate added, may not be fully explained as results of effects of ACTH on normal adrenals. The level of ACTH in these animals was not significantly higher than that of control animals. In accordance with previous studies, however, there were wide variations between different animals (18) and the results of the determinations of ACTH may therefore *per se* not exclude a slightly elevated level of ACTH in the treated animals. The excretion of free cortisol in urine was not increased in the treated animals and there was only a slight and statistically insignificant increase in plasma cortisol. In spite of this, there was an increase in adrenal cytochrome P-450 of about 60% and in increase in total side-chain cleavage of exogenous cholesterol of about 150%. Conversion of endogenous cholesterol was affected much less than that of exogenous cholesterol. Surprisingly, in this case the cholesterol content of adrenal mitochondria from treated animals was not very different from that of adrenal mitochondria from

control animals. Whether the difference between cholesterol side-chain cleavage of exogenous and endogenous cholesterol observed is a specific effect of ascorbate or not cannot be evaluated from the results of the present work (cf. below).

Inhibitory effect of ascorbate on cholesterol side-chain cleavage activity

That ascorbate inhibits cholesterol side-chain cleavage activity in the adrenals was clearly shown in the *in vitro* experiments. Thus, added ascorbate in a concentration of more than 1 mmol/l had an inhibitory effect on side-chain cleavage of both exogenous and endogenous cholesterol. Similar results have been reported by Shimizu (3) and by Sulimovici and Boyd (5). Since, according to the present and previous works (3, 4), the concentration of ascorbate in the normal adrenals is within the inhibitory range, it seems possible that inhibition by ascorbate is of some importance under *in vivo* conditions. The subcellular distribution of ascorbate is not known, however, and different parts of the cell may have widely different concentrations of ascorbate.

The inhibitory effect of ascorbate might explain part of the effects observed in animals with a moderate scurvy. The difference between side-chain cleavage activity of exogenous cholesterol in mitochondria from these animals and control animals decreased when isolated cytochrome P-450 was used as source of enzyme (Table 1). A possible explanation is that the mitochondrial fraction from normal guinea pigs contains some ascorbate, which inhibits cholesterol side-chain cleavage activity. This inhibition is released when using mitochondrial fraction from scorbutic guinea pigs and, as a consequence, cholesterol side-chain cleavage increases. It seems likely that most ascorbate in the mitochondrial fraction is lost in the isolation of cytochrome P-450. Thus there will be little or no difference in catalytic activity due to ascorbate content between preparations of cytochrome P-450 from adrenals of control and scorbutic guinea pigs. The finding that the inhibitory effect of added ascorbate on cholesterol side-chain cleavage activity was different in mitochondrial fraction from control animals and animals fed the scorbutogenic diet is also in accordance with the hypothesis that inhibitory ascorbate might be present in mitochondrial fractions from control animals. Since it is likely that some ascorbate is also lost from the mitochondria in the preparations, it is possible that the differences in side-chain cleavage capacity between mitochondria from adrenals of normal and scorbutic guinea pigs is higher *in vivo* than *in vitro*.

The above considerations might be valid in the side-

chain cleavage of exogenous cholesterol. The situation might, however, be somewhat different in the hydroxylation of endogenous cholesterol. Thus, in contrast to side-chain cleavage of exogenous cholesterol, the stimulatory effect of moderate scurvy on side-chain cleavage of endogenous cholesterol was small or absent.


In principle, the explanation could be that less endogenous cholesterol is available for the enzyme system when using mitochondria from scorbutic animals. The total cholesterol content of these mitochondria was, however, similar to that of mitochondria from control animals. Ascorbate deficiency might affect compartmentation of cholesterol within the mitochondria with consequences for side-chain cleavage of endogenous cholesterol. There is, however, no direct evidence for this hypothesis.

In view of the fact that moderate scurvy increased side-chain cleavage activity of exogenous cholesterol much more than that of endogenous cholesterol without affecting the excretion of cortisol in urine, it is tempting to suggest that hydroxylation of endogenous cholesterol *in vitro* better reflects the situation *in vivo* than hydroxylation of exogenous cholesterol. In the situation *in vivo*, however, there seems to be a continuous supply of cholesterol, mainly from the circulation (19, 20). It seems probable that such cholesterol is hydroxylated in a similar way as cholesterol added *in vitro*. Further work with the present technique under different conditions is needed, however, before it can be stated whether hydroxylation of exogenous or endogenous cholesterol is the best parameter for evaluation of the situation *in vivo*.

Nature of ascorbate inhibition of cholesterol side-chain cleavage

The inhibitory effect of ascorbate on cholesterol side-chain cleavage activity might be bound to adrenodoxin, adrenodoxin reductase, or cytochrome P-450. Since 11 β - and 18-hydroxylation of deoxycorticosterone utilize the same type of electron transport chain as cholesterol side-chain cleavage (for review, see ref. 21) and since only the latter hydroxylation was inhibited by ascorbate, it may be concluded that the effect of ascorbate is associated with the cytochrome P-450. Sulimovici and Boyd (5) showed that incubation of a cytochrome P-450 fraction with ascorbate was followed by loss of the heme iron. However, this mechanism seems to be of less importance under *in vivo* conditions. Thus the concentration of ascorbate necessary for loss of iron was considerably higher than that necessary for inhibition of cholesterol side-chain cleavage.

Is there a need for ascorbate in any step in steroidogenesis other than conversion of cholesterol into pregnenolone?

The increase in weight of the adrenals as a consequence of the scorbutogenic diet is interesting. Even in animals in which no increase in plasma cortisol was observed, the increase in weight of the adrenals was similar to that observed with the ACTH-treated animals. The increase in adrenal weight in animals fed the scorbutogenic diet was not due to edema, since there was a parallel increase in the amount of cytochrome P-450. The combination of adrenal hyperplasia, high side-chain cleavage activity towards exogenous cholesterol, and normal or only slightly increased output of cortisol seems to suggest that ascorbate might be of importance for more than the first step in steroidogenesis. There is no direct evidence for such a hypothesis, however. In any case, it is evident from the present work as well as from the work by Hodges and Hotston (7) that ascorbate cannot be obligatory for any of the different steps involved in the biosynthesis of cortisol. Thus a normal plasma level of cortisol can be maintained in spite of a markedly reduced level of ascorbate in the adrenals. Furthermore, in advanced scurvy with an increased level of ACTH, plasma cortisol is markedly increased. 

The skillful technical assistance of Miss Britt-Marie Mannerberg is gratefully acknowledged. This work was supported by the Swedish Medical Research Council (project 03X-3141) and a grant from Karolinska Institutets Forskningsfonder to Dr. A. Kallner.

Manuscript received 19 August 1977 and in revised form 19 December 1977; accepted 2 March 1978.

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