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35. Structural Correlates of Steroid Structures

EFFECT OF ACTH ON CHOLESTEROL AND STEROID SYNTHESIS IN ADRENOCORTICAL TISSUES

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Summary—Previous studies have established that under normal conditions, adrenal HMG-CoA reductase activity is higher in hamsters than in rats and humans. The hamster reductase activity follows a diurnal rhythm corresponding to that of plasma ACTH and glucocorticoids [*Endocrinology* **107** (1980) 215] but not to that of aldosterone. ACTH treatments to hamsters increased reductase activity after a latency of 60 min; this enhancement was prevented by cycloheximide [*J. steroid Biochem.* **24** (1986) 325]. Immunotitration and immunoblotting studies confirmed that ACTH caused an increase in reductase protein synthesis. In rats, long-term (1–9 days) and short-term (3 h) treatments with ACTH also induced increase in adrenal HMG-CoA reductase activity and reductase protein. In the presence of iodoacetamide and inhibitors of proteolytic enzyme, a main specific band of enzyme was evinced in the area of 102 ± 6 kDaM_r, by Western blotting, for both hamster homogenate and microsomal preparations (*Endocrinology*, **120** (1987)). Similarly M_r values were found with rat adrenal preparations. The concentration of mRNA, analyzed using the c-DNA pRed-10 coding for the Chinese hamster ovary reductase, was increased in adrenals of hamsters treated with ACTH. The reductase mRNA levels also fluctuated during the day in parallel with those of reductase activity and reductase protein. In conclusion, these results indicate that ACTH and other conditions inducing a change in hamster adrenal HMG-CoA reductase activity provoke parallel changes in reductase mRNA and reductase protein content. ACTH acts on the adrenal reductase of species synthesizing large as well as small quantities of cholesterol, thus indicating the general importance of this hormonal control.

INTRODUCTION

Cholesterol is the precursor of steroid hormones produced in the adrenal gland [1]. This substrate originates either from the uptake of plasma LDL by cell membrane receptors [2], from esterified cholesterol stored in liposomes or from *de novo* synthesis. The physiological significance of adrenal cholesterol synthesis is greatest in animal species that store it in smallest amounts, such as the hamster [3, 4], and is less in species such as the rat and the human [4] that have large stores of this substance. The biosynthetic pathways leading to the formation of cholesterol in the liver have been much studied. Moreover, it is well known that HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase is the key regulatory enzyme of the liver cholesterogenesis [5]. Many mechanisms have been proposed to explain how this regulation is effected at the molecular level such as (a) enzyme inactivation by phosphorylation–dephosphorylation [6, 7], (b) conversion product inhibition [8], (c) allosteric effect involving the level of reducing thiol groups in cells [9–11], (d) synthesis and degradation of the reductase itself [12–17]. More recently, HMG-CoA reductase activity has been demonstrated in the adrenal gland of many animal species [4]; this activity was found to be elevated in species capable of synthesizing much cholesterol (hamster, ox) and weaker in species (rat, human) producing less of this substance. In addition, diurnal rhythm of HMG-CoA reductase activity was reported for hamster [1, 18] and rat [19] adrenals. Moreover, the administration of adrenocroticotropin (ACTH) to hamsters induced reductase activity in a dose-dependent manner [20]. A long-term enhancing effect of ACTH was also reported for rat adrenal reductase activity [21]. It has also been found that ACTH induced HMG-CoA reductase activity in bovine adrenocortical primary cell cultures [22].

In this study we observed changes in adrenal reductase activity and enzyme protein levels occuring after ACTH treatment, and, also over the course of the dark–light cycle and we have attempted to correlate this with the reductase mRNA level.

MATERIALS AND METHODS

Materials

Mevalonic acid, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, $NADP^+$, dithiothreitol (DTT) cycloheximide, leupeptin, phenylmethylsulfonyl fluoride (PMSF) aprotinin and guanidine hydrochloride were purchased from Sigma. HMG-CoA was obtained from P.-L. Biochemicals. [^{125}I]Protein-A (0.7 μCi/μg) DL-3-hydroxy-3-methyl[3-^{14}C]glutaryl coenzyme A (55 mCi/mmol), DL-[5-^{3}H]mevalonate (6.7 Ci/mmol), were obtained from New England Nuclear. Reverse transcriptase RAV-2, restriction enzyme Pst I and labeled

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nucleotides [^{32}P]dATP, [^{32}P]dGTP, [^{32}P]dCTP (3000 Ci/mmol) were purchased from Amersham. Reagents for the analysis of plasma ACTH by radioimmunoassay were from Immuno Nuclear Corp. Agarose, ultrapure, DNA grade was obtained from Bio-Rad, unlabeled nucleotides from Boehringer Mannheim, glyoxal from Anachemia, low-melting-point ultrapure agarose and NACS (ion exchange resin) from BRL, and the oligonucleotides mixture pd(N_6) from Pharmacia. The corticotropin preparation used was Duracton (R) from Nordic Pharmaceuticals and Synacthen (R) Depot from Ciba. All other chemicals were of analytical grade.

Animals

Male Syrian golden hamsters weighing 100 ± 10 g, were purchased from Charles River Canada Inc., and kept for 3 weeks before experimentation in an isolated room with a controlled light–dark cycle (lights on, 0600–1800 h or lights on 1800–0600 h). Male Long Evans rats weighing 250 ± 25 g were obtained from our local breeding colony.

Treatments

For short-term experiments (up to 3 h) hamsters or rats were injected subcutaneously with 0.5 IU ACTH (Duracton) 100 g body weight (unless indicated otherwise) [20]; for long-term treatments 150 μg of Synacthen Depot was administered per rat [23]. As control, other animals received an equal volume of 0.15 M NaCl.

Tissue preparation

Animals were sacrificed at 1000 h, unless indicated otherwise. Adrenals were removed and homogenized in the following medium: 300 mM sucrose, 5 mM EDTA, 10 mM EGTA, 10 mM DTT, 0.6 M KCl, 1 mM PMSF, 0.1 mM leupeptin, 0.75 μg aprotinin, 50 mM imidazole, pH 7.0 [24]. Homogenates were subjected to differential centrifugation, and the pellet sedimenting between 8000 and 105,000 **g** was used as the microsomal fraction. When used, 50 mM NaF was added prior to homogenization [24].

HMG-CoA reductase assay conditions

Microsomes were suspended in a medium composed of 0.4 M KCl, 5 mM EDTA, 10 mM DTT, 1 mM PMSF, 0.1 mM leupeptin, and 50 mM imidazole, pH 8.0. A preincubation was performed at 37°C for 40 min; when used, 50 mM NaF or a rat liver phosphorylase phosphatase preparation equivalent to 100 μg of protein (post-ethanol fraction) [24] was added prior to the preincubation. The incubation was started by the addition of 20 mM G-6-P, (1 U/ml) G-6-P dehydrogenase, 3 mM $NADP^+$, 20,000 dpm [^{3}H]mevalonic acid and 200 μM of the substrate DL[3-^{14}C]HMG-CoA in a total volume of 200 μl. After a 5-min incubation at 37°C, DL[3-^{14}C]mevalonic acid formed was isolated and quantitated by TLC as previously decribed [24].

Immunoblotting

Adrenals were homogenized in a buffer similar to that of the reductase assay which contained no DTT, but 30 mM iodoacetamide. Homogenate and/or microsomal preparations were solubilized in 62.5 mM Tris–HCl buffer, pH 6.8, containing 2.3% sodium dodecyl sulfate (SDS), 10% glycerol and 8 M urea by heating for 90 s at 90°C. Electrophoresis was performed according to Laemmli[25], to a 8.0% polyacrylamide gel at pH 8.8. Proteins were transferred on nitrocellulose by Western blotting [26–28]. The nitrocellulose sheet was incubated with a rabbit antirat liver reductase antibody (from Dr G. C. Ness, Department of Biochemistry, University of South Florida) for 2 h at 25°C. After washings the complex was incubated in the presence of 5 μg [^{125}I]protein-A/30 ml. In control experiments, normal rabbit serum was substituted for the serum antibody. Specifically bound radioactivity was linear for the protein range used, for both homogenate and microsomal preparations. Two concentrations of protein were routinely applied on gels for immunoblotting studies. Nitrocellulose sheets were analyzed by radioautography and subsequently cut in 0.5 cm fractions to determine their radioactivity. After subtraction of background, results were expressed either in cpm/μg protein and/or in cpm/gland.

mRNA isolation

Adrenals were homogenized in 15 vol of a 7 M guanidine–HCl solution containing 20 mM iodoacetic acid, 1% lauroyl sarcosine and 1 mM EDTA, pH 5, and kept at −20°C. After several passages on ice through 21 **g** syringe needle and centrifugation at 10,000 **g** for 10 min at −10°C, to the supernatant was added 1/10 vol of 2.4 M ammonium acetate, pH 5.0, and 0.55 vol of ethanol at −20°C. The mixture was kept at −20°C for 1½ h and then at −70°C for 10 min followed by a 20-min centrifugation at 15,000 **g** at −10°C. The pellet was solubilized in 0.1 vol at room temperature (relative to the original volume of homogenization buffer) of 7 M guanidine–HCl, pH 7.0; this was followed by the addition of 1/20 vol of 2.4 M ammonium acetate and 0.55 vol of ethanol at −20°C. After 45 min the preparation was centrifuged at 5000 **g** for 15 min at −10°C. The pellet obtained was washed 3 times at room temperature with a solution of sodium acetate 3 M, pH 6, containing 1 mM EDTA and 0.1% lauroyl sarcosine. RNA was precipitated as described above and the pellet washed twice with 80% ethanol [29].

RNA electrophoresis and blotting

Total RNA was denatured with glyoxal and electrophoresed in 1% agarose in 0.01 M phosphate

buffer, pH 6.5 [30]. After electrophoresis the gel was blotted to nitrocellulose as described by Southern[31]. The paper was prehybridized for 16 h at 43°C and hybridized for 24 h at 43°C according to Liscum *et al.*[16]. Blots were probed with a [^{32}P]labeled reductase c-DNA prepared from pRed-10 [32] kindly provided by Dr M. S. Brown, The University of Texas Health Science Center at Dallas.

The [^{32}P]labeled c-DNA probe was prepared by digestion of pRed-10 with Pst I followed by electrophoresis on low-melting agarose 1%; after staining with ethidium bromide, the band containing the 1.2 kDa fragment was cut, melted, and the insert was extracted from agarose using a NACS ion exchange resin column. The c-DNA probe was nick-translated in the presence of [^{32}P]dATP and [^{32}P]dGTP, to a specific activity of $1–4 \times 10^8$ cpm/μg and used in hybridizations at a concentration of 2 ng/ml (10^6 cpm/ml).

Radioautograms were obtained by exposing the blots to CURIX RP1 film with Cronex Lightning plus enhancing screens (E.I. Dupont). The relative amount of reductase mRNA determined by densitometry scanning of the blot was linearly related to total mRNA (0–20 μg).

Blots were also analyzed with a [^{32}P]ribosomal probe. Total hamster adrenal mRNA was deposited on an oligo (dt)-cellulose column [33]; the poly (A)-RNA fraction was collected and used to make the ribosomal probe. Poly(A)-RNA (0.5 μg) was added to a 50 mM Tris–HCl buffer, pH 8.1, containing 2 mM DTT, 5 mM $MgCl_2$, 40 mM KCl and 250 μg of oligodeoxynucleotides. After boiling for 5 min, the preparation was quickly chilled on ice and then incubated at 14°C for 30 min. Forty μM of each of unlabeled d-ATP, d-GTP, d-TTP, 25 μCi of [^{32}P]dCTP and 50 units of reverse transcriptase were then added and the mix incubated at 37°C for 1 h. The reaction was stopped by boiling for 10 min in the presence of 1% SDS and 0.2 M NaOH, followed by the addition of 0.13 M acetic acid and STE (10 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA, pH 8.0). The labeled probe was separated from unincorporated dNTPs by chromatography using a column of Sephadex G-50. For hybridation, 60,000 cpm/ml were used. Blots were dehybridized at 65°C for 2 h in a 5 mM Tris–HCl buffer, pH 8.0, containing 0.2 mM EDTA, 0.05% pyrophosphate, 0.1X Denhardt's [0.002% (w/v) Ficoll, 0.002% (w/v) BSA and 0.002% (w/v) polyvinylpirrolidone]. Prehybridization, hybridization and washings were made as described above for the pRed-10 probe. Densitometric quantification of radioautograms was made; data obtained with the ribosomal probe were used to correct for manipulation losses.

Hormone assays

Plasma corticosteroid and aldosterone were determined as previously described [34]. Plasma ACTH levels were analyzed by a specific radioimmunoassay without extraction. When the synthetic ACTH Duracton was added to a pool of plasma, the level of ACTH recovered was 76%.

Protein determination

The protein content of samples used for electrophoresis and for the reductase assay was determined by the method of Bradford as modified by Rubin and Warren [35].

RESULTS

Hamster adrenal HMG-CoA reductase activity and plasma hormonal level

Figures 1(A) and 1(B) show the effect of the time of day on plasma corticosteroid level and adrenal HMG-CoA reductase activity; in agreement with previous reports [1, 18] these two parameters possess a diurnal rhythm. There is a parallelism between these two profiles and that of plasma ACTH (Fig. 1C) but not with that of aldosterone (results not shown). HMG-CoA reductase activity is often reported as total activity when microsomes are exposed to phosphorylase phosphatase, or as expressed activity when prepared and analyzed in the presence of sodium fluoride, an inhibitor of phosphorylase phosphatase. Figure 1(D) shows the ratio of expressed/total activity of adrenal preparations from hamsters sacrificed at different times during the day. The ratio (%) increased from 9.6% at 1000 h to 13.9% at 2200 h when activity was maximal, and decreased thereafter. Although statistically significant $0.02 > P > 0.01$ this increase was quite small.

Immunotitration studies

To further test if the observed incrementation of adrenal reductase activity was due to activation of an inactive enzyme or to *de novo* synthesis, preparations from hamsters which had been submitted to various experimental conditions were analyzed by immunotitration. First, we determined that the presence of rat liver anti-HMG-CoA reductase antibody in incubation media inhibited hamster adrenal reductase activity in a dose-dependent manner (Fig. 2). With substitution of the antibody by an equivalent volume of normal rabbit serum, there was no inhibition. Table 1 shows that for a fixed quantity of antibody chosen from the straight part of the curve (Fig. 2), the same loss of enzyme activity was found for preparations originating from hamsters treated with ACTH or with 0.15 M NaCl, although total activity was increased 1.86-fold at 120 min after ACTH treatment. In the presence of antibody a similar loss in enzyme activity was observed for preparations of animals sacrificed either at 2000 h or at 1000 h, although a 2.2-fold increase in total activity was observed for the 2000 h preparation, also indicating an increase in protein content rather than an activation of a pre-existent inactive enzyme.

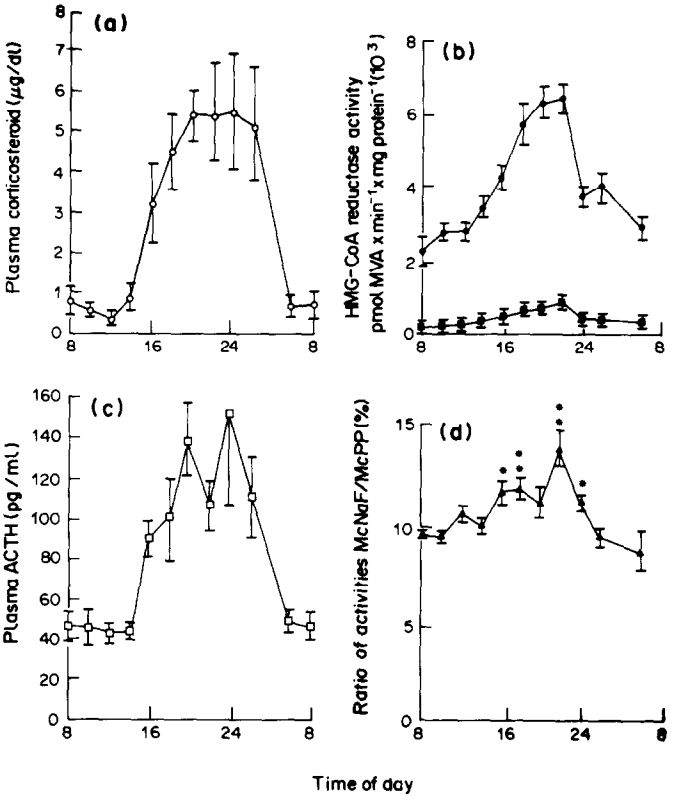

Fig. 1. Diurnal variation of hamster plasma corticosteroids and ACTH levels and of HMG-CoA reductase activity in adrenal microsomes. (A) and (C): Plasma corticosteroids and ACTH were determined by radioimmunoassay. (B): ●, Microsomes prepared, preincubated and incubated in a buffer containing NaF (McNaF); ■, microsomes prepared in the presence of NaF but preincubated without it and in the presence of phosphorylase phosphatase (McPP), as described in Materials and Methods. (D): Ratio (%) of values from (B). Number of animals used per point curve: 3 × 4 animals. Values are means ±SE of 3 experiments. $^{*}P < 0.05$; $^{**}P < 0.02$ compared with the 1000 h sample (t-test).

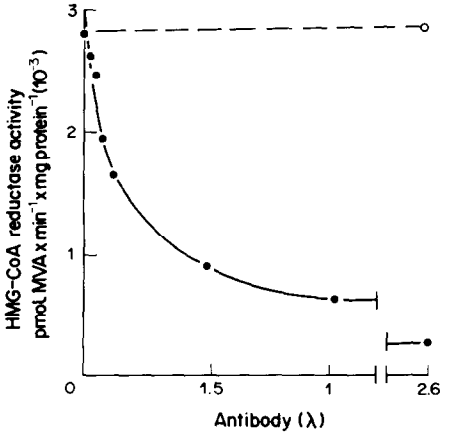

Fig. 2. Inactivation of hamster adrenal HMG-CoA reductase by the addition of increasing amounts of rat liver anti-(HMG-CoA reductase). Microsomal preparations (140 μg) were preincubated at 37°C for 30 min and then cooled at 25°C prior to the addition of antibody or normal rabbit serum, followed by a 20-min incubation at 25°C. HMG-CoA reductase activity was then determined as described under Materials and Methods. ●—●. In the presence of antibody; O--O, in the presence of normal rabbit serum. Data are means of duplicate determinations.

Kinetic studies

Experimental conditions were selected so that less than 10% of the substrate HMG-CoA was transformed during the reaction. The calculated K_m value from Lineweaver–Burk plots was 5.44 μM (S) HMG-CoA for the preparation of control animals sacrificed at 1000 h. No differences in reductase K_m values were found between preparations originating from ACTH-treated animals or from hamsters sacrificed either at 1000 h or 1900 h, although V_{max} was increased in experimental groups (results not shown).

Western blotting

We also analyzed the level of HMG-CoA reductase protein in hamster adrenal by immunoblotting. Figure 3 shows radioautograms of whole homogenate and microsomal preparations after Western blotting: in both cases a main band of reductase protein complexed with [^{125}I]protein A was seen in the area of M_{rs} slightly greater than that of the standard 97.4-kDa phosphorylase b; traces were also observed at 90 and 62 kDa.

Table 2 compares the radioactivity content of this

Table 1. Immunoinhibition of HMG-CoA reductase activity in hamster adrenal microsomes

Treatment (time after treatment)	Enzyme activity/mg microsomal protein (pmol MVA/min per mg protein)	P	Loss of enzyme activity produced by a fixed quantity of antibody (pmol MVA/min per mg protein)	P
(A) NaCl 0.15 M (15 min)	2601 ± 158	—	163 ± 14.0	—
ACTH (15 min)	3305 ± 214	NS	168 ± 16.8	NS
ACTH (120 min)	4848 ± 319	< 0.001	163 ± 5.5	NS
(B) — (Animals sacrificed at 1000 h)	3202 ± 152	—	123 ± 6.7	—
— (Animals sacrificed at 2000 h)	7095 ± 222	< 0.001	193 ± 39.2	NS

Microsomal preparations were divided and incubated with or without a fixed quantity (0.1 λ) of rabbit anti rat liver HMG-CoA reductase. ACTH administered: 0.5 IU/100 g body wt. Values are means ±SE of 3 different experiments performed in triplicate. *P*, level of significance calculated according to the Student *t*-test.

main band (shown in Fig. 3) for adrenal preparations of hamsters under various experimental conditions. ACTH induced, 135 min after its administration, 1.53- and 2.81-fold increase in reductase content of homogenate and microsomal preparations, respectively, whereas the microsomal reductase activity was increased 2.99-fold. Compared to animals sacrificed at 1100 h (under light for 4 h), in the B series of experiments, maximal increases of 1.92, 1.96- and 1.76-fold were observed at 2000 h (2 h after dark) for homogenate and microsomal reductase content and microsomal reductase activity respectively. This table shows that when microsomal reductase activity was increased, the quantity of reductase protein was also increased for both microsomal and homogenate preparations.

We also performed experiments with the rat to determine if ACTH would have similar effects on the adrenal reductase of a species synthesizing *de novo* much less cholesterol than the hamster. Short-term as well as long-term effects were observed. We found a 2.8-fold increase in microsomal HMG-CoA reductase activity at 180 min post ACTH treatment, and a 2.1-fold increase in reductase protein (97.4 kDa) measured in homogenates as expressed in cpm/gland. Chronic ACTH treatment to rats for 3 and 9 days resulted in 11- and 16-fold increases in reductase protein (whole homogenate), whereas microsomal activity was enhanced 10- and 15-fold respectively. Similar results were found when Western blotting was performed on microsomes (Results not shown).

Table 2. Effect of ACTH and of the time of day when hamsters were sacrificed on their adrenal HMG-CoA reductase activity and content

Experiment	Reductase activity (pmol/mg prot per min)* Microsomes	P	Reductase content (cpm[^{125}I]/μg prot)*† Homogenate	P	Microsomes	P
Series A						
Control	1535 ± 116	—	30.4 ± 1.7	—	92 ± 16	—
135 min after ACTH treatment	4589 ± 450	< 0.001	46.4 ± 1.8	< 0.001	258.6 ± 32	< 0.01
Series B [46]						
Time of sacrifice 1100 h	2396 ± 112	—	27.3 ± 6.4	—	27.1 ± 47	—
1900 h	3779 ± 450	< 0.01	48.8 ± 5.2	< 0.02	47.1 ± 2.5	< 0.01
2000 h	4602 ± 934	< 0.02	53.6 ± 2.6	< 0.01	47.9 ± 8.4	< 0.05
2100 h	3065 ± 378	< 0.05	42.9 ± 6.1	< 0.05	43.5 ± 2.7	< 0.01

*Values are mean ±SD of three experiments. *P*, level of significance calculated according to the Student *t*-test.
†The radioactivity of the main band after immunoblotting (as shown in Fig. 3) was used for the calculation of the reductase content of both homogenate and microsomal preparations. ACTH administered: 0.5 IU/100 g body wt.

Reductase mRNA

To determine whether these increases in reductase activity and reductase protein were due to corresponding changes in mRNA levels, the relative levels of adrenal HMG-CoA reductase mRNA were determined by hybridization. Total mRNA was subjected to electrophoresis in agarose gels and transferred on to nitrocellulose paper. Figure 4(A) shows that the pRed10 probe hybridized to a single band. In order to normalize these results the nitrocellulose sheets were washed and rehybridized with the poly(A)-ribosomal probe. Figure 4(B) shows radioautogram of a typical experiment. It also shows that reductase mRNA hybridized at the same level as the 28S mRNA.

Fig. 5 compares the effect of light and darkness on

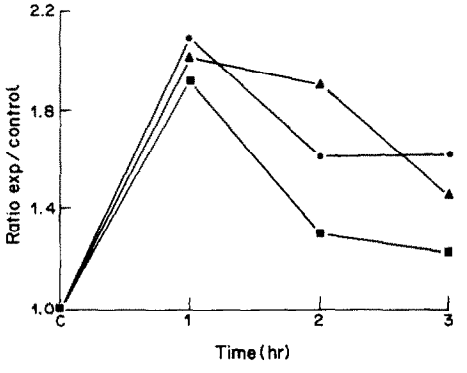

Fig. 5. Effect of darkness on hamster adrenal HMG-CoA reductase activity, reductase content and reductase mRNA. Control animals were kept on a normal cycle (lights on 0600 h, lights off 1800 h and sacrificed at 1000 h. Experimental groups were kept on an inverted light cycle (lights on 1800 h, lights off 0600 h) and sacrificed 1, 2 and 3 h of darkness respectively. Hamsters were sacrificed on the same day but different sets of animals were used for each type of analysis. ■, Microsomal reductase activity; ▲, reductase content based on the radioactivity of the main band (per mg protein after Western blotting of whole homogenate; ●, reductase mRNA was determined by Southern blotting using the cDNA pRed10, and appropriate corrections were made after determination of respective ribosomal RNA on nitrocellulose sheet with a ribosomal probe.

reductase activity, reductase protein, and reductase mRNA. Values obtained from preparations of animals sacrificed at 1000 h, 4 h after the lights were turned on, served as control. It can be seen that 1 h after the lights were turned off (inverted light cycle), reductase mRNA was increased 2.1-fold whereas reductase activity and reductase protein (homogenate) were increased 1.92- and 2.02-fold respectively. These ratios decreased thereafter but remained still high after 3 h of darkness.

Table 3 shows that the level of reductase mRNA increased after ACTH administration to hamsters. In this particular experiment ACTH induced 2.1-, 2.4- and 3.7-fold increases in mRNA levels 60, 120 and 180 min post treatment respectively. At the same time, enhancements in reductase activity and reductase protein were also observed; maximal values were found 180 min post treatment with 2.4- and 1.7-fold increases for the reductase activity and content respectively.

DISCUSSION

In this work we showed that the variations in hamster adrenal HMG-CoA reductase activity paralleled both the reductase protein, and reductase mRNA level. In agreement with previous reports from this laboratory [1, 18], we observed a diurnal rhythm in hamster plasma corticosteroid. As expected, we found a similar diurnal variation for plasma ACTH. Also in agreement with our previous reports, we observed a parallelism between plasma corticosteroid levels and those of adrenal microsomal HMG-CoA reductase activity [1, 18]. The profile and the height of diurnal cycles varied depending on the season; highest values being found during spring and summer time, and lowest values at the beginning of winter. This explains differences in basal control values. It is therefore important to compare groups of animals having the same age, the same sex, kept under the same housing conditions and sacrificed on the same day.

The ratio of expressed/total HMG-CoA reductase activity, increased by 4.3% between 1000 and

Table 3. Effect on adrenal HMG-CoA reductase activity, content, and mRNA levels of ACTH to hamster: a time study

Time of sacrifice after treatment (min)	Ratio experimental/control		
	Reductase activity	Reductase protein*	Reductase mRNA
0	1.00	1.00	1.00
60	1.65	1.27	2.12
120	1.81	1.55	2.37
180	2.35	1.72	3.78

Hamsters were sacrificed on the same day but different sets of animals were used for each type of analysis. ACTH administered: 0.5 IU/100 g body wt.

*Based on radioactivity incorporated in the main band after immunoblotting of whole homogenates. Other analyses were done as described under Materials and Methods.

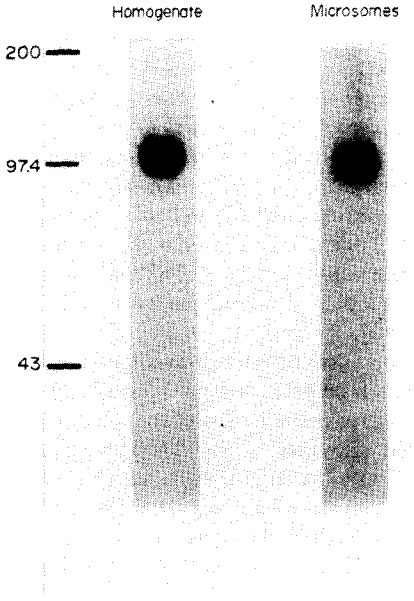

Fig. 3. Immunoblotting analyses of hamster adrenal HMG-CoA reductase preparations. Fresh adrenal samples were homogenized in a medium containing iodoacetamide. An aliquot of the homogenate was kept for electrophoresis and the rest centrifuged to obtained microsomes. Homogenates and microsomes were solubilized in a buffer containing 2.3% SDS and 8% urea and then submitted to electrophoresis followed by immunoblotting using a specific antireductase serum. The equivalent of 0.014 gland was applied to each gel for homogenate and the equivalent of 0.046 gland for microsomes.

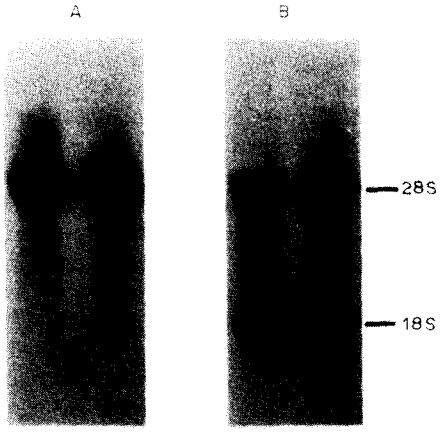

Fig. 4. Analysis of adrenal HMG-CoA reductase mRNA. Total RNA was isolated from hamster adrenal, denatured with glyoxal, electrophoresed on 1% agarose gels, and transferred to nitrocellulose as described under Materials and Methods. The nitrocellulose was exposed for 24 h at 43°C with the ^{32}P-labeled reductase c-DNA insert of p-Red 10, washed and analyzed by radioautography. The nitrocellulose was dehybridized, rehybridized with a ^{32}P-labeled ribosomal RNA probe. (A): Hybridization with the ^{32}P-labeled reductase c-DNA. (B): Hybridization with the ^{32}P-labeled ribosomal probe.

2200 h, in microsomal preparations from hamster adrenal. Although this increment is statistically significant, it is rather small and may not be important; it does not allow us, however, to completely rule out the participation of a control mechanism involving phosphorylation–dephosphorylation. In other experiments, we found that the expressed/total activity was not increased during the first 60 or 180 min post ACTH administration to animals [24], although adrenal reductase activity was increased. Such a control mechanism, by phosphorylation–dephosphorylation, was reported for the rat liver HMG-CoA reductase activity by Easom and Zammit [36]; indeed, these authors found that the ratio between the expressed activity and the total activity varied markedly in a diurnal pattern between 28 and 80% at the nadir and peak, respectively, of the cycle.

Our results show that the rat liver anti-HMG-CoA reductase antibody specifically cross-reacted with the hamster adrenal reductase. With this antibody, we determined that ACTH was inducing the quantity of reductase rather than activating an inactive enzyme. Indeed, the loss in activity between control and ACTH-treated animals would have been different in the latter situation. Similar studies performed on preparations obtained from hamsters sacrificed at 1000 and 2000 h also indicated that the increase in reductase activity observed at 2000 h was equally due to increased protein synthesis (Table 1). In rat liver, Dugan *et al.*[37] also reported that changes in enzyme activity, (increase or decrease) within 1 h following cholesterol feeding or glucagon or mevalonate administration to diabetic rats, were accompanied by no change in the specific activity of the enzyme, as determined from the quantity of enzyme activity inactivated by a fixed quantity of antibody.

The K_m [5.44 μM (S)HMG-CoA] for hamster adrenal microsomal reductase preparations was similar to those reported for intestine homogenate (4.1 μM) [38] and for radish seedling membrane (4.7 μM) [39]. A wide range of values (1–35 μg) were reported for liver preparations [40–42] but low values were found (1–3 μM) for solubilized and partly purified preparations [43–45]. The K_m for the hamster adrenal reductase is, also, very similar to that we reported for the human adrenal (4–5 μM) [9]. The fact that we found no real differences in reductase K_m values between preparations originating from ACTH-treated animals or from hamsters sacrificed either at 1000 or 1900 h also indicates that increases in reductase activity, observed under these conditions, might be due to increased reductase protein synthesis.

The M_r for the hamster adrenal [46] is slightly higher than that deduced from the DNA sequence derived from UT-cells [8]; it is similar to the value reported for the rat liver reductase by Ness *et al.*[47], but higher than that we reported for the human adrenal [9]. It is noteworthy that this value was for hamster reductase prepared in buffers containing iodoacetamide and inhibitors of proteolytic enzyme. This 102 ± 6-kDa M_r band [46] might thus correspond, *in vivo*, to the smallest non-degraded reductase unit. With rat adrenal preparations, a main band was also observed in the area of M_r slightly higher than that of phosphorylase b, but another one was present in the area of 175 kDa M_r, even in the presence of iodoacetamide in homogenization buffer to block SH groups (results not shown). A very small quantity of this higher-M_r material was also observed for hamster adrenal preparations [46]. The meaning of these observations is not clear at present. Using inactivation by radiation and immunoblotting techniques, other laboratories have found two populations of reductase for rat liver, with M_r of about 100 and 200 kDa respectively [48, 49]; they concluded that the liver reductase consists of a noncovalently linked dimer of the M_r 97-kDa enzyme subunit.

In expressing immunoblotting results on the basis of cpm/gland, only 40–50% of reductase protein was found in microsomes [46], indicating the presence of more than one pool of adrenal reductase. This is in agreement with results of Keller[50] that a substantial fraction of normal rat liver reductase is not microsomal.

The conclusion from our immunoblotting studies is that when hamster adrenal HMG-CoA reductase activity increased, the mass of reductase enzyme also increased and vice versa. This phenomenon was observed after ACTH treatments (Tables 2 and 3) and also when animals were sacrificed at different times during the day (Table 2).

Our studies also revealed that ACTH as well as darkness induced parallel changes in the levels of adrenal reductase activity, reductase protein and reductase mRNA (Table 3 and Fig. 5); our findings are in agreement with those of Liscum *et al.*[16] that increases of reductase protein in rat liver occurred because of increases in the amount of its mRNA. Moreover, Clarke *et al.*[14] reported a parallelism between the diurnal rhythms of reductase protein and reductase mRNA in rat liver. We also found 12 times more reductase mRNA in a human adrenal carcinoma than in normal adrenal (unpublished observations) when the reductase activity of the carcinoma was considerably more elevated than that of the normal adrenal [9].

Our results on the short-term enhancing effect of ACTH on rat adrenal reductase activity are in agreement with those of Balasubramaniam *et al.*[2]. They did not find, as we do however, a sustained effect in reductase activity by chronic ACTH administration. By injecting, once daily, the long-acting form of ACTH, Synacthen Depot, we found an increase in reductase activity as well as in reductase protein, 3 days and 9 days post treatment, respectively. One explanation for this difference could be the form of ACTH (ACTHAR) the other

group used. The fact that ACTH acts on the adrenal reductase of species synthesizing large as well as small quantities of cholesterol generalizes the importance of this hormonal control.

Although there is a parallelism between adrenal HMG-CoA reductase activity, reductase protein, and reductase mRNA, we do not yet know if these changes are due to a direct effect of ACTH, or to an indirect action through lowering of the level of endogenous substrates, such as that of cholesterol or related compounds. In this respect, we have reported that the level of adrenal free cholesterol was significantly decreased in glands of hamsters kept under physiological conditions and sacrificed at 1900 h compared to control sacrificed at 1100 h [51]. We have also observed that the administration of ACTH to hamsters at a dosage of 0.5 IU/100 g body weight produced a significant decrease in adrenal free cholesterol content; with lower doses of ACTH, however, such a decrease was not observed [20]. In addition, low levels of free and esterified cholesterol were found in rat adrenal after chronic ACTH treatments [21], and in a human adrenal carcinoma having a high HMG-CoA reductase activity [9, 23]. Moreover, the concentration of adrenal free cholesterol was increased in adrenals of hamsters treated with cycloheximide or with cycloheximide and ACTH, when HMG-CoA reductase was diminished compared to saline-treated controls. Also in agreement with these observations, is a report on primary cultures of bovine adrenocortical cells, which suggests that ACTH may control *de novo* cholesterol synthesis by increasing cholesterol flux into steroid [22]. More work will be necessary to clarify this point.

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