Chem. 237, 1469.
Leiberman, S., Dobriner, K., Hill, B. R., Fieser, L. F., and Rhoads, C. P. (1948), *J. Biol. Chem. 172*, 263.
Leiberman, S., Praetz, B., Humphries, P., and Dobriner, K. (1953), *J. Biol. Chem. 204*, 491.
Lewis, J. R., and Shoppee, C. W. (1955), *J. Chem. Soc.*, 1365.
Magendantz, H. G., and Ryan, K. J. (1964), *J. Clin. Endocrinol. Metab. 24*, 1155.
Marrian, G. F., and Butler, G. C. (1944), *Biochem. J. 38*, 322.
Okada, M., Fukushima, D. K., and Gallagher, T. F. (1959), *J. Biol. Chem. 234*, 1688.
Reynolds, J. W. (1963), *Proc. Soc. Exptl. Biol. Med. 113*, 980.
Reynolds, J. W. (1966), *Steroids 7*, 261.
Ruse, J. L., and Solomon, S. (1966a), *Biochemistry 5*, 1065.
Ruse, J. L., and Solomon, S. (1966b), *Biochemistry 5*, 1072.
Ryan, K. J. (1959), *J. Biol. Chem. 234*, 2006.
Schubert, K., and Wehrberger, K. (1965), *Endokrinologie 47*, 290.
Slaunwhite, W. R., Karsay, M. A., Hollmer, A., Sandberg, A. A., and Niswander, K. (1965), *Steroids 6, Suppl. II*, 211.
Weichselbaum, T. E., and Margraf, H. W. (1960), *J. Clin. Endocrinol. Metab. 20*, 1341.
YoungLai, E., and Solomon, S. (1966), *Federation Proc. 25*, 281.

Adrenocorticotrophin-Induced Changes in the Steroidogenic Activity of Adrenal Cell-Free Preparations*

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ABSTRACT: After incubation of rat adrenal quarters *in vitro* with adrenocorticotrophic hormone (ACTH), the steroidogenic activity of subsequently derived homogenates is enhanced. This *induced* effect of ACTH in the homogenates occurs despite maximal quantities of a reduced triphosphopyridine nucleotide (TPNH)-generating system and does not appear to be *directly* due to ACTH or 3′,5′-adenosine monophosphate (3′,5′-AMP), and the inductive process (in the intact tissue) is blocked by puromycin. The effect appears to be largely owing to a factor(s) recovered in the 60,000*g* supernatant which interacts with adrenal mitochondria and enhances total corticosterone production, conversion of [^{14}C]cholesterol to corticosterone, and cholesterol side-chain cleavage (relative to control supernatant). The ACTH-induced supernatant factor is present in the macromolecular fraction excluded by Sephadex G-25, and the factor seems to be $(NH_4)_2SO_4$ precipitable, heat labile, and trypsin labile. Cyclic AMP induces comparable changes in the cholesterol side-chain-cleaving activity of rat adrenal supernatant, as does ACTH in the human adrenal cortex. The control rat adrenal supernatant inhibited cholesterol side-chain cleavage.

The factor(s) responsible for inhibition was present in the fraction excluded by Sephadex G-25, and appeared to be $(NH_4)_2SO_4$ precipitable and heat labile. This factor was observed in adrenal tissue from the rat, dog, rabbit, pig, and man, but not in extraadrenal rat tissues. It is unknown whether ACTH stimulates steroidogenesis in the cell-free system by counteracting the control inhibitory factor, or *via* a stimulatory factor which functions independently of the inhibitor.

The biological events that occur between the initial interaction of ACTH[1] with adrenal tissue and its subsequent effect on steroidogenesis have remained obscure. This ignorance is largely due to the ineffectiveness of ACTH in cell-free systems. Although studies with inhibitors of protein synthesis in more intact systems have suggested a role for protein synthesis during the steroidogenic action of ACTH (Ferguson, 1963; Farese, 1964; Garren *et al.*, 1965), the correct interpretation of these studies is doubtful since it is not certain that the inhibitors were operative *via* effects on protein synthesis. In the present investiga-

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[1] Abbreviations used: ACTH, adrenocorticotrophic hormone; KRB, Krebs–Ringer bicarbonate buffer; TPN^+ and TPNH, oxidized and reduced triphosphopyridine nucleotides; SN, supernatant fluid; AMP, adenosine monophosphate; ATP, adenosine triphosphate; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

tion we have attempted to study more directly the possibility that a protein mediator may be involved during ACTH action, by utilizing Ferguson's (1963) observation that limited exposure of adrenal tissue to ACTH *in vitro* induces a continued enhancement of steroidogenic activity. This observation had indicated that an ACTH-induced factor(s) (*e.g.*, a cofactor, corticosteroid precursor, or protein) had accumulated, and could, in turn, stimulate steroidogenesis, even after new induction of the factor had been blocked by inhibitors of protein synthesis. We have attempted to identify and characterize this steroidogenic factor by inducing its formation in relatively intact adrenal tissue *in vitro* by ACTH, and studying its action in a cell-free system. It was hoped that this experimental approach would provide a more effective means for defining some of the intermediate events during ACTH action on steroidogenesis, and permit more critical testing of the hypothesis that a protein mediator is involved.

Experimental Procedures

Male Sprague–Dawley rats weighing approximately 250 g were obtained from the Holtzman Co. Adrenals were rapidly removed after sacrifice (by guillotine), placed in ice-cold 0.9% saline, trimmed, and cut into quarters. One or two quarters from each adrenal were then distributed to two or four incubation flasks, respectively.[2] A number of quarters were accumulated (according to the needs of the experiment) and the quarters were routinely preincubated for 30 min at 37° under 95% O_2 plus 5% CO_2 in KRB containing glucose (10 mM). After preincubation, the medium was decanted, fresh KRB was added, and the adrenal sections were incubated for 30 min at 37° with or without ACTH (final medium concentration = 1 IU/ml; Wilson's oxycel-purified ACTH was used except where indicated). After incubation the media were poured off for subsequent determination of corticosterone. (A two- to fivefold increase owing to ACTH was regularly observed.) The tissue fragments were then blotted, weighed, and homogenized with 15 strokes in 0.154 M KCl (either 70 or 140 mg of tissue wet wt/ml) using a Potter-Elvejhem homogenizer equipped with a motor-driven Teflon pestle.

The homogenates or subcellular fractions were assayed for steroidogenic activity by incubation in the system described by Koritz and Péron (1959). Basically, this system included in a final volume of 2 ml: homogenate or subcellular fractions derived from 28 mg of adrenal tissue,[3] 40 μmoles of $NaHCO_3$ (final pH 7.4 under 95% O_2 plus 5% CO_2 at 37°), 33 μmoles of $CaCl_2$, 2.1 mg of glucose 6-phosphate; 1.6 mg of TPN^+, and 0.154 M KCl. In some experiments, where twice as much tissue was employed, all ingredients were increased proportionally. As described by Péron and Robidoux (1964), the subcellular fractions required for steroidogenic activity included the sediment obtained by centrifugation for 15 min at 15,000*g* and 4° (Spinco, Model L-2) and the SN obtained by subsequent centrifugation of the 15,000*g* SN at 60,000*g* for 15 min. These fractions were isolated as described by these authors, except that a preliminary centrifugation of the homogenate at 265*g* for 5 min was not employed, and the sediments were washed as indicated in the text.

In place of lengthy dialysis, low molecular weight substances were separated from proteins (and other macromolecular substances) in the 60,000*g* SN by passage through Sephadex columns (G-25, coarse; approximate dimensions, 0.6 × 20 cm; external volume, approximately 2 ml) previously equilibrated with 0.154 M KCl at 4°. Doubly concentrated 60,000*g* SN (0.8 ml) was applied, and the eluted protein fraction was easily identified in the exclusion volume by its tan color and slight cloudiness. The volume of the protein fraction was measured and then adjusted to 1.6 ml with 0.154 M KCl. With this technique, 95% of the eluted protein was recovered in this fraction, and low molecular weight substances were removed (checked by adding ^{14}C tracers) from the protein fraction with the following efficiencies: glycine (95%) and ribose (97%), corticosterone (90%), ATP (90%), progesterone (85%), and pregnenolone (82%). Little or no corticosterone was detectable in the SN after Sephadex treatment, and the Sephadex-treated SN from 225 mg of control or ACTH-treated adrenals did not contain sufficient progesterone or pregnenolone to affect the specific activity of added [^{14}C]progesterone or [^{14}C]-pregnenolone (2 μc added to the SN, extracted with $CHCl_3$–ethanol (3:1), chromatographed on thin layer silica gel plates with $CHCl_3$–acetone (9:1), eluted, and quantitated by 240-mμ absorption or colorimetry, respectively). The protein concentrations of fractions prepared in parallel from control and ACTH-stimulated adrenals were routinely checked, and were found to be quite comparable (not differing by more than 5% from the mean, except in a few instances where results were not used).

Corticosterone was usually determined on duplicate aliquots of the incubation mixtures by the sulfuric acid fluorescence method of Silber *et al.* (1958). In some experiments, where indicated, corticosteroids were measured by the blue tetrazolium method of Elliott *et al.* (1954). Cortisol was measured by the method of Porter and Silber (1950). Proteins were determined by the method of Lowry *et al.* (1951) with isolated rat adrenal protein as the standard. Cholesterol was determined by the modified method of Schoenheimer and Sperry as described by Hawk *et al.* (1954). Pregnenolone was measured by the method of Koritz (1962).

Conversion of ^{14}C precursors to corticosterone was determined after the following: addition of a tracer (1 μc) of [1,2-^3H]corticosterone to each flask at the

[2] Thus, approximately equal amounts of tissue from each adrenal were present in the control and treatment group(s).

[3] The protein concentrations of homogenates and subcellular fractions from control and ACTH stimulated were routinely measured and were not found to differ by more than 5%.

TABLE I: Steroidogenic Activity of Homogenates from Control and ACTH-Stimulated Adrenal Quarters.[a]

	Adrenal Quarter Incubn (medium, corticosterone, μg/100 mg of adrenal tissue)		Homogenate Incubations (μg of corticosterone/flask)							
	Control	ACTH	Control				ACTH			
Expt	30 min	30 min	0 min	15 min	30 min	60 min	0 min	15 min	30 min	60 min
1	1.2	8.8	1.4	19.9	43.7	46.2	3.1	17.9	56.6	53.5
2	5.5	12.5	3.9	12.3	18.8	19.3	2.8	23.2	38.1	45.9
3	2.0	3.7	3.9	12.0	37.3	30.2	4.2	18.5	55.4	27.7
4	2.3	9.5	0.8	11.2	30.0	37.0	2.8	16.2	29.4	29.4
5	0.4	2.4	0.8	14.0	21.8	34.7	1.7	25.8	31.4	22.7
6	1.1	4.8	4.5	23.8	44.0	42.6	8.1	35.6	45.4	72.5
7	0.8	8.1	4.3	14.1	20.5	23.4	8.3	22.4	28.3	26.3
8	1.3	11.3	3.4	25.8	25.8	68.9	3.4	15.7	32.5	91.0
9	1.6	7.8	4.1	15.0	26.2	53.6	7.0	28.0	45.3	56.6
10	1.1	6.2	0.9	21.3	28.8	56.3	7.9	43.2	40.7	60.2
11	1.5	5.4	2.3	19.8	47.1	53.1	6.2	26.0	52.8	67.2
12	0.9	2.2	0.5	19.1	26.0	37.0	2.4	29.0	30.1	31.2
13	1.1	3.3	1.7	13.8	29.8	32.0	4.5	13.9	39.2	41.8
14	0.9	4.0	2.5	25.0	32.1	36.0	6.0	26.0	41.9	50.0
Mean	1.6	6.4	2.5	17.7	30.9	40.7	4.9	24.4	40.5	48.3
Mean difference ± SE (ACTH *vs.* control)		4.8 ± 0.8						6.7 ± 2.0	9.6 ± 1.6	7.5 ± 3.4
P		<0.001						<0.01	<0.001	<0.05

[a] After 30-min incubation of adrenal quarters with or without ACTH (1 U/ml of medium), adrenal homogenate derived from 28 mg of tissue was incubated for the time period indicated. Values of corticosterone production are shown on the left for the adrenal quarters, and on the right for homogenates derived from these quarters. Results of 14 experiments are shown. *P* was determined by t-test evaluation of the mean difference at 13 deg of freedom.

end of incubation; extraction of the flask contents first with two volumes of isooctane (which was discarded) and then with 2.5 volumes of $CHCl_3$; washing the $CHCl_3$ extract with three-tenths volume of 0.1 N NaOH; paper chromatography of the dried $CHCl_3$ extract in the system benzene–hexane–80% methanol (2:1:1); elution of the corticosterone area; and repeated chromatography on the dried eluate in the same system or the Bush 5 system (Bush, 1952) after the addition of 0.1 mg of carrier corticosterone. Recovery losses were corrected for, and radiochemical purity of the product was judged by the $^{14}C:^{3}H$ ratio. Chromatography was repeated until a constant $^{14}C:^{3}H$ ratio was obtained, and, in pilot experiments, this ratio was maintained in the mother liquor and crystals after adding approximately 1 ml of ethanol saturated with carrier corticosterone and recrystallizing by cooling. Eluates were dried, dissolved in toluene, PPO, and POPOP (see Farese and Reddy, 1963), and counted in a Nuclear-Chicago liquid scintillation counter. At the settings employed, the counting efficiencies for ^{14}C and ^{3}H were approximately 50 and 15%, respectively, approximately 0.03–0.05% of the ^{3}H counts per minute registering in the ^{3}H channel carried over to the ^{14}C channel, and approximately 35–40% of ^{14}C counts per minute registering in the ^{14}C channel carried over to the ^{3}H channel. Carry-over counts per minute were corrected for, and all samples were counted long enough for an error of less than ±3% (1 std dev). Zero-time samples carried through all procedures were devoid of ^{14}C counts per minute in the final corticosterone product, except, as indicated, when the cholesterol pool of the 15,000*g* sediment had been labeled with [4-^{14}C]cholesterol during a preliminary incubation. However, even in the latter experiments, zero-time counts per minute were quite low, and the increase in counts per minute paralleled the increase in corticosterone over the course of the incubation.

The conversion of cholesterol to pregnenolone in the present incubation system was measured by the

method of Ichii *et al.* (1963) wherein [^{14}C]isocaproic acid, liberated by cleavage of the side chain of [26-^{14}C]-cholesterol, is obtained in the steam distillate of the acidic lipid fraction. The recovery of [^{14}C]isocaproic acid through the extractions and steam distillation was 54 ± 8% (mean plus and minus standard deviation) in 21 separate experiments, and replicate recoveries in each experiment were usually within 5% of the mean. The incubation itself did not affect recovery, and all losses of isocaproic acid appeared to occur during steam distillation. After paper chromatography of the steam distillate of several pooled incubation samples in the system described by Brown (1950), the only substance detectable by radioactive scanning (Nuclear-Chicago Actigraph III) had the same chromatographic mobility as authentic isocaproate.[4] Zero-time values (counts per minute, including background, in the volatile, and acidic lipid fraction of unincubated samples) usually varied between 40 and 60 cpm, and were routinely subtracted from the values of incubated samples. Samples were dissolved in dioxane containing naphthalene, PPO, and POPOP (Butler, 1961), and the counting efficiency was approximately 70%.

The following materials were employed: oxycel-purified ACTH (Wilson) (act. 100 IU/mg), chromatographically purified ACTH (Sigma) (act. 200 IU/mg), 3′,5′-AMP (Schwarz), sodium glucose 6-phosphate (Nutritional), NaTPN (Nutritional), glucose 6-phosphate dehydrogenase (Calbiochem or General Biologicals), [4-^{14}C]cholesterol (Nuclear-Chicago) (sp act. 24.6 μc/μmole), [26-^{14}C]cholesterol (Nuclear-Chicago) (sp act. 24 μc/μmole), [4-^{14}C]pregnenolone (Nuclear-Chicago) (sp act. 24 μc/μmole), [4-^{14}C]progesterone (Nuclear-Chicago) (sp act. 21.7 μc/μmole), [1,2-^{3}H]-corticosterone (New England Nuclear) (sp act. 1040 μc/μmole), [1-^{14}C]isocaproate (Nuclear Equipment) (sp act. 0.57 μc/μmole), trypsin (Sigma), soybean trypsin inhibitor (Sigma), and puromycin (Nutritional).

Results

Effects of ACTH on the Steroidogenic Activity of Adrenal Homogenates. After 30-min incubation of adrenal quarters with ACTH, homogenates derived from these quarters were more active for corticosterone formation than the corresponding homogenates derived from similarly incubated control adrenal quarters. The absolute values of steroid production in the homogenates varied considerably during the course of these experiments, and hormonal effects, although regularly observed, were best evaluated statistically by analysis of the mean difference between groups. Table I shows the results of 14 experiments. At each time period of homogenate incubation, significant stimulatory effects of ACTH were observed. (Comparable results were also obtained when corticosteroid produc-

[4] The radioactive product of similar incubation systems has also been identified as isocaproic acid by Staple *et al.* (1956), Lantos *et al.* (1964), Ichii *et al.* (1963), and Constantopoulos *et al.* (1966).

TABLE II: Effects of ACTH in Quartered Rat Adrenals with or without Addition of Glucose 6-Phosphate and TPN$^+$.[a]

	μg of Corticosterone/100 mg of Tissue							
	Without Glucose-6-PO_4 and TPN$^+$				With Glucose-6-PO_4 and TPN$^+$			
Incubn Type	15 min	30 min	60 min	90 min	15 min	30 min	60 min	90 min
Control	0.7 ± 0.2	1.1 ± 0.3	2.2 ± 0.3	2.1 ± 0.5	5.3 ± 0.5	11.6 ± 1.6	26.5 ± 6.3	33.8 ± 2.0
ACTH	2.1 ± 0.2	5.8 ± 0.8	11.6 ± 0.5	16.2 ± 1.9	5.4 ± 0.9	15.1 ± 1.4	41.7 ± 3.2	47.3 ± 8.4

[a] Adrenal quarters from 16 rats were distributed to 16 flasks and, following routine preincubation, were incubated in 2 ml of KRB with or without 5 mg of glucose 6-phosphate and 4 mg of TPN$^+$. Where indicated ACTH was present at a final medium concentration of 1 U/ml. Mean values (plus and minus variation) of two comparable experiments are shown.

TABLE III: Comparison of Steroidogenic Activities of Control *vs.* ACTH Homogenates with Variable Levels of Glucose 6-Phosphate or TPN^+.[a]

Homogenate Type	μg of Corticosteroid/Flask											
	A. TPN^+ Concentration (mM)						B. Glucose-6-PO_4 Concentration (mM)					
	0	0.16	0.33	0.67	1	2	0	0.6	1.2	2.4	3.5	7.0
Control	0.5	4.6	7.4	7.6	7.5	7.6	1.7	15.3	17.2	17.0	17.0	11.7
ACTH	1.3	5.4	10.0	9.9	10.6	8.7	3.3	20.3	25.6	26.7	23.5	19.4

[a] After 30-min incubation of adrenal quarters with or without ACTH (1 U/ml of medium), adrenal homogenates derived from 28 mg of tissue were incubated for 30 min in the cell-free system. In expt A, corticosterone was measured by the acid–fluorescence method and glucose 6-phosphate concentration was held constant at 2.1 mg/flask (or 3.5 mM). In expt B (unrelated to A) corticosteroid was measured by the blue tetrazolium method and the TPN^+ concentration was held constant at 1.6 mg/flask (or 1 mM). Comparable results were obtained in repeat experiments.

tion was measured by the blue tetrazolium method.) In general, reaction rates in the homogenate were linear or near linear during the first 30 min, and the greatest relative difference between control and ACTH homogenates was usually observed at 30 min, and was, on the average, approximately 30–40%.

The relative and absolute ACTH effect observed in homogenate incubations was as great (or nearly as great) as that observed when control and ACTH-stimulated adrenal quarters were incubated in the presence of maximal amounts of glucose 6-phosphate plus TPN^+. This may be surmised from the results of Table II, which also shows the effect of these cofactors on the base-line steroid production rate of rat adrenal quarters. Although the absolute ACTH effect was similar in the supplemented and unsupplemented incubations of adrenal quarters, the relative effect was considerably reduced in the former. Along these lines, it may be noted (Table I) that the absolute increase in homogenate steroid production owing to ACTH (at 30-min incubation), was much greater than that occurring after incubation of adrenal quarters for 30 min.

While the above results suggested that it may be possible to directly study the steroidogenic factor(s) induced by ACTH, they provided no insight concerning the nature of the factor(s). A number of studies were conducted to evaluate several important possibilities. Since TPNH generation has often been suggested as the mechanism whereby ACTH stimulates steroidogenesis (for a review, see Hilf, 1965), this was evaluated. As shown in Table III, ACTH effects on homogenate activity were demonstrable regardless of the amount of added glucose 6-phosphate or TPN^+, and it seemed unlikely that TPNH availability could be limiting under the usual conditions of these incubations. (In support of this, supplemental glucose 6-phosphate dehydrogenase did not affect the rate of stereoidogenesis in this system.) Since 3′,5′-AMP (Roberts *et al.*, 1965) and ACTH (McKerns, 1964) have been reported to have *direct* effects on steroid biosynthesis, these substances were tested in the present homogenate system, and were found to have no demonstrable effects. To evaluate the possibility that a protein may be responsible for the ACTH effect in the homogenate system, studies were also conducted with puromycin. As shown in Table IV, when puromycin (at concentrations which inhibit amino acid incorporation by more than 95%) was present during the incubation of adrenal quarters, the inductive effect of ACTH on subsequent homogenate activity was largely inhibited.

Subcellular Localization of the ACTH Effect. To further characterize the changes induced by ACTH,

TABLE IV: Effects of Puromycin on Inductive Action of ACTH on Steroidogenic Activity of Adrenal Homogenates.[a]

Expt	Homogenate Type	μg of Corticosterone/Flask			
		0 min	15 min	30 min	60 min
A	Control	4.2	17.9	31.4	31.1
	ACTH	5.0	29.4	41.7	59.4
	Puromycin	3.9	15.7	34.2	43.7
	ACTH + puromycin	3.1	21.0	34.2	40.9
B	Control	4.4	23.6	44.0	42.8
	ACTH	6.0	35.6	52.0	72.8
	Puromycin	4.8	19.2	30.0	47.2
	ACTH + puromycin	4.8	22.4	34.4	52.0

[a] In each experiment adrenal quarters were distributed to four flasks and incubated with or without ACTH (1 U/ml of medium) and/or puromycin (1 mM). Homogenates were then prepared and incubated for the times indicated in the cell-free system.

the homogenates were at first simply centrifuged at 15,000*g* for 15 min. The SN's and sediments thus obtained from control and ACTH homogenates were then assayed for steroidogenic activity by incubating with the opposite complementary subcellular fraction (*i.e.*, sediment or SN, respectively) derived from another group of control adrenals. In eight of nine such experiments, stimulatory effects of ACTH were found in the 15,000*g* SN (mean per cent change relative to control SN activity = +36%; $P < 0.005$). In six experiments, effects were also apparent in the unwashed 15,000*g* sediment (mean per cent change relative to control sediment activity = +20%; $p < 0.025$). Thus, a clear-cut segregation of the ACTH effect into the SN or sediment was not always apparent. However, since the effects were somewhat greater and more regularly observed in the SN, and since this fraction seemed easier to study, further attention was given to this fraction.

As stated previously, the essential components of the incubation system include a mitochondrial-rich 15,000*g* sediment and the SN obtained at 60,000*g*.

TABLE V: Steroidogenic Activity of Control *vs.* ACTH 60,000*g* Supernatant.[a]

Expt	Tissue Incubn Components	μg of Corticosterone/Flask 0 min	15 min	30 min	60 min
1	Control 60,000*g* SN + control 15,000*g* sediment	2.1	8.0	12.8	12.0
	ACTH 60,000*g* SN + control 15,000*g* sediment	4.0	13.2	16.4	14.6
2	Control 60,000*g* SN + control 15,000*g* sediment	1.2	2.8	5.6	8.8
	ACTH 60,000*g* SN + control 15,000*g* sediment	2.1	4.0	8.0	15.6

[a] After 30-min incubation with or without ACTH (1 U/ml of medium), adrenal quarters were homogenized and centrifuged at 15,000*g* for 15 min. The SN's (supernatants) obtained were centrifuged for another 15 min at 60,000*g*. Control sediment obtained at 15,000*g* was resuspended in KCl and recentrifuged. Incubation flasks contained the tissue components derived from 28 mg of adrenal tissue along with the other usual ingredients of the cell-free system. Incubation times are shown.

These subcellular fractions were prepared from control and ACTH homogenates, and, as shown in Table V, the ACTH 60,000*g* SN was more active than control SN when both were incubated with control 15,000*g* sediment.

Characterization of the Effects of ACTH on 60,000g SN Activity. To examine the possibility that the ACTH-induced stimulatory factor(s) present in the 60,000*g* SN may be macromolecular, Sephadex (G25) columns were employed (see Methods) to remove most low molecular weight substances. Protein fractions thus obtained were incubated with control 15,000*g* sediment, and, as shown in Table VI, the fractions obtained from ACTH-stimulated adrenals were more active in supporting steroidogenesis than the corresponding control fractions.[5]

The above results suggested that the effect of ACTH was not simply due to the presence of steroid precursors, since: (1) pregnenolone, progesterone, and corticosterone were largely removed (see Methods) from the protein fraction by Sephadex chromatography; and (2) the corticosteroid formed in this system is reportedly derived solely from mitochondrial-free cholesterol (Péron and Robidoux, 1964). To evaluate this further, control and ACTH Sephadex-treated 60,000*g* SN's were incubated with control 15,000*g* sediment whose cholesterol pool had been labeled with [4-^{14}C]cholesterol during a prior incubation of adrenal quarters (this technique was employed to minimize problems of solubility and pool sizes, and to permit testing of the effects of the SN on the conversion of "endogenous" mitochondrial precursors, in this case labeled during the prior incubation to corticosterone). As shown in Table VII, conversion of ^{14}C precursor to [^{14}C]corticosterone was greater in the presence of the ACTH Sephadex-treated 60,000*g* SN.

To further define the step(s) in the steroid biosynthetic pathway affected by the ACTH-induced factor(s), control and ACTH Sephadex-treated 60,000*g* SN's were incubated with control 15,000*g* sediment and [4-^{14}C]pregnenolone or [4-^{14}C]progesterone. As shown in Table VIII, these substances were avidly converted to [^{14}C]corticosterone, but there were no demonstrable differences between results obtained with control or ACTH SN (despite the fact that the ACTH SN enhanced the production of corticosterone from endogenous precursors).

Effects of Control vs. ACTH Sephadex-Treated

[5] Sephadex-treated 60,000*g* SN was in most cases as active as the untreated SN in supporting corticosterone formation in the cell-free system. Maximal steroidogenic rates occurred when equivalent amounts (*i.e.*, based on the original wet weight of tissue) of SN and sediment were incubated together. The somewhat lower absolute rates of steroidogenesis shown in Table VI, as compared to previous experiments, were not due to the use of Sephadex-treated SN; rather, it reflects the unexplained shifts in absolute steroidogenic rates noted in adrenal quarter incubations as well as homogenate incubations in the present experiments. Of greater importance is the relative ACTH effect which is comparable to that observed in previous experiments (*e.g.*, Table I).

TABLE VI: Steroidogenic Activity of Protein Fractions from Control and ACTH 60,000*g* Supernatants.[a]

	μg of Corticosterone/Flask					
	Control SN Protein Fraction + Control 15,000*g* Sediment			ACTH SN Protein Fraction + Control 15,000*g* Sediment		
Expt	0 min	30 min	60 min	0 min	30 min	60 min
1	0.5	2.4	5.2	0.5	5.1	8.3
2	0.1	2.8	5.3	0.1	5.5	5.3
3	0.3	2.3	3.4	0.3	4.3	4.6
4	0.6	2.1	3.0	0.3	3.5	8.7
5	0.8	6.8	4.4	0.5	7.1	5.5
6	0.1	2.1	5.4	0.2	2.7	6.3
7	0.2	3.8	5.8	0.4	5.1	7.7
8	0.3	6.4	12.0	0.2	7.3	15.4
Mean	0.4	3.6	5.6	0.3	5.1	7.8
Mean difference ± SE (ACTH *vs.* control)				−0.1	1.5 ± 0.3	2.2 ± 0.6
P				NS	<0.005	<0.025

[a] Experiments conducted as in Table V except the 60,000*g* SN's were passed through Sephadex (G-25) columns (see Methods) and protein fractions (approximately 1 mg of protein for control and ACTH) thus derived were employed in the incubations. Mean results of eight experiments are shown. *P* was determined by t-test evaluation of the mean difference at 7 deg of freedom. NS = not significant.

TABLE VII: Conversion of ^{14}C Precursor to Corticosterone as Affected by Control *vs.* ACTH Sephadex-Treated 60,000*g* Supernatant.[a]

	Cpm Converted to Corticosterone					
	Control SN Protein Fraction + Control 15,000*g* Sediment			ACTH SN Protein Fraction + Control 15,000*g* Sediment		
Expt	15 min	30 min	60 min	15 min	30 min	60 min
1	296	756	1536	376	989	1681
2	584	796	1676	1020	1517	1704
3	—	936	1752	—	1011	2056
4	196	412	729	297	729	1417
5	—	312	501	—	348	712
6	291	575	1142	258	712	1170
7	208	364	752	312	624	1288
Mean	315	593	1155	452	847	1433
Mean difference ± SE (ACTH *vs.* control)				138 ± 110	254 ± 86	278 ± 95
P				NS	<0.05	<0.05

[a] Experiments conducted as in Table VI except the 15,000*g* sediment were from adrenal quarters previously incubated for 90 min with 12.5 μc of [4-^{14}C]cholesterol (dissolved in 0.15 ml of ethanol and added to 10 ml of KRB). Incubation flasks contained: washed, labeled 15,000*g* sediment from 28 mg of adrenal tissue (containing approximately 2.5 mg of protein and 20,000–40,000 cpm); SN protein fractions from 28 mg of tissue (containing approximately 1 mg of protein for control and ACTH); and other usual additions. Incubations were conducted for the times indicated, following which 1 μc of [1,2-^{3}H]corticosterone was added, and corticosterone was isolated by chromatographing twice in the system benzene–hexane–80% methanol (see Methods). Zero-time flasks contained, on the average, 61 cpm as [^{14}C]-corticosterone. Mean results of seven experiments are shown. *P* was determined by t-test evaluation of the mean difference at 6 deg of freedom. NS = not significant.

TABLE VIII: Conversion of Various Precursors to Corticosterone in the Presence of Control *vs.* ACTH Sephadex-Treated 60,000*g* Supernatant.[a]

Precursor	Conversion to Corticosterone: Control SN Protein Fraction + 15,000*g* Sediment	Conversion to Corticosterone: ACTH SN Protein Fraction +15,000*g* Sediment
Endogenous (μg/flask)	1.6 (range = 1.5–1.8, *n* = 3)	4.8 (range = 3.7–6.1, *n* = 3)
[4-^{14}C]Pregnenolone (%)	39	43
[4-^{14}C]Progesterone (%)	70	71

[a] Protein fractions from control and ACTH 60,000*g* SN's (supernatants) were obtained after chromatography on Sephadex (G-25) columns. The 15,000*g* sediment was obtained from control adrenal quarters and washed once by resuspension and recentrifugation. Incubation was for 30 min and each flask contained: 15,000*g* sediment (approximately 2.5 mg of protein) from 28 mg of control adrenal tissue; control or ACTH Sephadex-treated 60,000*g* SN (approximately 1.0 mg of protein) from 28 mg of adrenal tissue; 0.625 μc of ^{14}C precursor dissolved in 0.05 ml of ethanol; and other usual additions. After incubation 1 μc of [1,2-^{3}H]corticosterone was added and the flask contents were extracted and subjected to paper chromatography (see Methods) in the system benzene–hexane–80% methanol. The corticosterone area was eluted and part was used for corticosterone measurement (by acid fluorescence) and the values were corrected for recovery of ^{3}H. The remainder was divided, 0.1 mg of carrier corticosterone was added, and one-half was rechromatographed twice in the same system and one-half twice in the Bush 5 system. The conversion values shown are the means of closely agreeing results obtained through both chromatographic systems. The ^{14}C:^{3}H ratio was constant between the second and third chromatographies, and through subsequent recrystallization from ethanol. Comparable results were obtained in a repeat experiment.

60,000g SN's on Cholesterol Side-Chain Cleavage. The above results suggested that the ACTH-induced steroidogenic factor(s) may be more effectively studied by focusing on the conversion of cholesterol to pregnenolone. Accordingly, 15,000*g* sediment, previously labeled by a prior incubation of control adrenal quarters with [26-^{14}C]cholesterol,[6] was incubated alone or with control or ACTH Sephadex-treated 60,000*g* SN. As shown in Table IX, the time course of [^{14}C]isocaproate formation (or cholesterol side-chain cleavage) was similar to that of steroidogenesis and conversion of [^{14}C]cholesterol to corticosterone (see above). In addition, the yield of [^{14}C]isocaproate was *decreased* by the presence of control Sephadex-treated 60,000*g* SN.

Table X shows the results of 11 experiments in which control and ACTH Sephadex-treated 60,000*g* SN's were tested for their effects on cholesterol side-chain cleavage. A consistent pattern was found; the production of [^{14}C]isocaproate was greatest when sediment was incubated with no added SN, least upon addition of control Sephadex-treated 60,000*g* SN, and intermediate upon addition of ACTH Sephadex-treated 60,000*g* SN. (In expt 8, the comparable results obtained with chromatographically purified ACTH suggested that the effects were not due to impurities in the cruder preparation.)

Since the yield of [^{14}C]isocaproate could be influenced by changes in the recovery or metabolism of [^{14}C]isocaproate, changes in the size or specific activity of the metabolically active cholesterol pool, or changes in the conversion of the intermediate, isocaproaldehyde, to isocaproic acid (see Constantopoulos *et al.*, 1966), as well as by side-chain cleavage of [26-^{14}C]cholesterol *per se*, these possibilities were evaluated. The following pertinent findings were obtained. (1) $^{14}CO_2$ production (trapped in Hyamine in center wells after acidification of the media at the end of incubation) was quite limited and uninfluenced by the presence or absence of control or ACTH Sephadex-treated SN during the incubation. (2) Comparable results were obtained when NaCN (1 mM) was present during the incubation. (3) Upon incubation of 0.1 μc of [^{14}C]isocaproate in the three incubation systems, the recovery of [^{14}C]isocaproate was uninfluenced by the presence of either control or ACTH Sephadex-treated SN. (In fact, there was no appreciable effect of the incubation itself on the recovery of [^{14}C]isocaproate, which was approximately 54%.) (4) Sephadex-treated SN did not displace (or cause "leakage" of) [26-^{14}C]cholesterol from the 15,000*g* sediment to the 15,000*g* SN to any greater extent than that occurring without added SN (approximately 5–15%). (5) Addition of nonradioactive cholesterol to the incubation only slightly inhibited [^{14}C]-isocaproate formation (*e.g.*, 8% inhibition with 25 μg of cholesterol). (6) The control and ACTH Sephadex-treated 60,000*g* SN's contained, on the average, much lesser amounts of free cholesterol (7.8 and 8.3 μg, respectively, in the SN from 56 mg of tissue). (7) The specific activity of the labeled cholesterol (isolated by thin layer chromatography of the neutral lipid extract in the system described by Avigan *et al.*, 1963), presumably serving as the substrate for

[6] Chromatography of ether–ethanol extracts of the labeled 15,000*g* sediment on thin layer silica gel plates in the system benzene–ethyl acetate (5:1) (see Avigan *et al.*, 1963) revealed that virtually all radioactivity (determined by scanning) was in the unesterified cholesterol area.

TABLE IX: Time Course of [^{14}C]Isocaproate Formation Resulting from Cholesterol Side-Chain Cleavage.[a]

Incubation Components	[^{14}C]Isocaproate Yield (cpm/flask)				
	5 min	10 min	20 min	40 min	60 min
15,000*g* sediment alone	79 ± 9	124 ± 1	172 ± 15	277 ± 18	290 ± 11
15,000*g* sediment + control Sephadex-treated 60,000*g* SN	66 ± 2	89 ± 9	127 ± 0	219 ± 11	250 ± 13

[a] Washed, labeled 15,000*g* sediment was from adrenal quarters previously incubated for 90 min with 10 μc of [26-^{14}C]cholesterol (dissolved in 0.1 ml of propylene glycol and 5 ml of KRB). Protein fractions were obtained after passage of the SN through Sephadex (G-25) columns. Incubation flasks contained in a volume of 4 ml: 15,000*g* sediment (approximately 5 mg of protein) from 56 mg of adrenal tissue (containing approximately 50,000 cpm); where indicated, control Sephadex-treated 60,000*g* SN (approximately 1.7 mg of protein) from 56 mg of adrenal tissue; 2 units of glucose 6-phosphate dehydrogenase; and other usual additions. After incubation for the times indicated, the [^{14}C]isocaproate yield was determined. Shown here are the mean results plus and minus variation of duplicate incubation flasks.

the cleavage reaction, was not appreciably affected by the addition of control or ACTH Sephadex-treated SN. (8) The pattern of total steroid formation (pregnenolone plus 240-mμ absorbing lipid-extractable material) was virtually identical with that of [^{14}C]isocaproate formation obtained when the 15,000*g* sediment was incubated alone and with control or ACTH Sephadex-treated SN. (9) As reported by others (see above), isocaproaldehyde was virtually undetectable as a product of the cleavage reaction (method of Constantopoulos *et al.*, 1966) resulting from incubation of labeled 15,000*g* sediment alone or with control Sephadex-treated 60,000*g* SN. These results suggest that the pattern of [^{14}C]isocaproate formation can be directly correlated with cholesterol side-chain-cleaving activity.

Effects of Ammonium Sulfate Fractions from Control vs. ACTH 60,000g SN's on Cholesterol Side-Chain Cleavage. To study the possibility that the inhibitory effects of the control SN and the stimulatory effects of the ACTH SN (relative to control) may involve proteins, the SN's were subjected to $(NH_4)_2SO_4$ precipitation. As shown in Table XI, the redissolved, dialyzed precipitates,[7] obtained at 40% $(NH_4)_2SO_4$ saturation, affected cholesterol side-chain cleavage in a fashion comparable to that observed with the crude Sephadex-treated 60,000*g* SN's (*cf.* Tables X and XI).

Effects of 3',5'-AMP on Cholesterol Side-Chain-Cleaving Activity of the 60,000g SN. Cyclic-3',5'-AMP, which has been suggested (see Haynes and Berthet, 1957) to act as a mediator during ACTH action, was also tested in the present system. As shown by the representative experiments in Table XII, after incubation of adrenal quarters with 3',5'-AMP, both the Sephadex-treated 60,000*g* SN and the 0–40% ammonium sulfate fraction derived from the 60,000*g* SN enhanced cholesterol side-chain cleavage (as compared to the corresponding controls). These induced effects of 3',5'-AMP were quite comparable to those induced by ACTH.

In contrast to the induced effects of 3',5'-AMP, direct addition of this substance to the incubation of labeled 15,000*g* sediment (with or without added Sephadex-treated 60,000*g* SN) produced little or no effect on cholesterol side-chain cleavage.

Effects of Heat and Trypsin on the Cholesterol Side-Chain-Cleaving Activity of the 60,000g SN. As shown in Table XIII, brief heating at 100° for 3 min, or incubation at 37° for 30 min in the presence of trypsin, diminished the differences between the control and ACTH Sephadex-treated 60,000*g* SN's in their ability to affect cholesterol side-chain cleavage. This was due to both an increase in activity of the control SN and a decrease in activity of the ACTH SN. A comparable decrease in activity differences between control and ACTH 0–40% ammonium sulfate fractions was achieved simply by incubation at 37° for 30 min, and this too was effected by a decrease in activity of the ACTH fraction and an increase in the activity of the control fraction (Table XIV). The inhibitory activity of the control 0–40% ammonium sulfate fraction was lost after heating for 2 min at 60° (Table XIV).

Specificity of the Effects of Adrenal 60,000g Supernatant on Cholesterol Side-Chain Cleavage. In view of the possibly important role that the inhibitory factor(s) present in the control Sephadex-treated

[7] The variations in protein concentrations in the $(NH_4)_2SO_4$ fractions may have been due to the fact that in expt 1 and 3, the 60,000*g* SN's were freshly prepared and the pH during ammonium sulfate fractionation was approximately 5.4, whereas in expt 2 the 60,000*g* SN's had been frozen for several months at −20° and the pH was approximately 6.0. In other experiments in which freshly prepared 60,000*g* SN was used, protein concentrations were comparable to those in expt 1 and 3.

TABLE X: Effects of Control *vs.* ACTH Sephadex-Treated 60,000*g* Supernatant on Cholesterol Side-Chain-Cleaving Activity.[a]

Expt	Cpm in 15,000*g* Sediment (approximately)	[^{14}C]Isocaproate Yield (cpm/flask)		
		15,000*g* Sediment Alone I	15,000*g* Sediment + Control 60,000*g* SN Protein Fraction II	15,000*g* Sediment + ACTH 60,000*g* SN Protein Fraction III
1	47,000	472	282	364
2	148,000	2098	786	878
3	52,000	808	652	748
4	20,000	390	320	380
5	18,000	398	221	544
6	22,000	507	177	368
7	20,000	258	146	186
8	40,000	936	457	946
9	77,000	390	333	381
10	27,000	137	33	78
11	34,000	187	85	230
Mean	46,000	598	317	464
Mean difference ± SE	−281 ± 110 (II *vs.* I)		157 ± 43 (III *vs.* II)	
P	<0.05		<0.005	

[a] Washed, labeled 15,000*g* sediment was prepared as in Table IX. Control and ACTH SN protein fractions were obtained after passage of the SN's through Sephadex (G-25) columns. Incubation flasks contained: 15,000*g* sediment (approximately 5 mg of protein) from 56 mg of adrenal tissue; where indicated, protein fractions of the 60,000*g* SN (approximately 1.7 mg of protein) from 56 mg of adrenal tissue; 2 units of glucose 6-phosphate dehydrogenase; and other usual additions. Incubation was for 60 min after which [^{14}C]isocaproate formation was measured (see Methods). In many of the experiments there were closely agreeing duplicates. *P* was determined by the t-test evaluation of the mean difference between groups as indicated. In expt 8 the ACTH SN protein fraction was from adrenal quarters incubated with chromatographically purified ACTH (Sigma).

60,000*g* SN may play in the regulation of steroidogenesis, the specificity of this inhibitory effect was examined. As shown in Table XV, bovine serum albumin markedly

TABLE XI: Effects of Ammonium Sulfate Fractionation on Cholesterol Side-Chain-Cleaving Activity of Control *vs.* ACTH 60,000*g* Supernatant.[a]

Expt	[^{14}C]Isocaproate Yield (cpm/flask)					
	15,000*g* Sediment Alone	15,000*g* Sediment + Control $(NH_4)_2SO_4$ Fractions (% satn)			15,000*g* Sediment + ACTH $(NH_4)_2SO_4$ Fractions (% satn)		
		0–40	40–70	0–70	0–40	40–70	0–70
1	667 ± 52	364 ± 1 (0.17)	585 ± 10 (0.65)	503 ± 46 (0.81)	501 ± 23 (0.16)	533 ± 4 (0.72)	736 ± 8 (0.80)
2	519	180 (0.43)	256 (0.51)	—	327 (0.42)	302 (0.57)	—
3	505 ± 23	298 ± 24 (0.39)	418 ± 2 (0.82)	—	439 ± 57 (0.37)	417 ± 23 (0.82)	—

[a] The 60,000*g* SN's from control and ACTH-stimulated adrenal quarters were prepared as usual except in expt 3 where the SN was filtered through a short Sephadex (G-25) column to remove particulate lipids. A saturated solution of $(NH_4)_2SO_4$ in 0.154 M KCl was then added slowly with mixing at 0–4° to 40 or 70% saturation. After standing for 1.5–2 hr, precipitates were collected by centrifugation. To the supernatant obtained at 40% saturation, additional $(NH_4)_2SO_4$ was added to 70% saturation and the precipitate was collected. Precipitates were resuspended in several milliliters of 0.154 M KCl and dialyzed for 5 (expt 1 and 2) or 18 hr (expt 3) *vs.* 2 l. of 0.154 M KCl at 0–4°. Undissolved protein was removed by centrifugation in expt 3 (but not in expt 1 or 2). Protein concentrations were determined, and, after minor volume adjustments to equalize the concentrations of control and ACTH fractions, the fractions derived from 56 mg of adrenal tissue were incubated with equivalent amounts of control 15,000*g* sediment (labeled with [26-^{14}C]cholesterol and containing approximately 63,000, 77,000, and 31,000 cpm/flask in expt 1, 2, and 3, respectively). Other details of incubations are provided in Table IX. In expt 1 and 3, duplicate incubation flasks were employed and the mean results plus and minus variation are shown. The amount (in milligrams) of protein present in the $(NH_4)_2SO_4$ fraction added per incubation flask is shown in parentheses.

TABLE XII: Comparative Effects of ACTH and 3′,5′-AMP on the Cholesterol Side-Chain-Cleaving Activity of the 60,000*g* Supernatant.[a]

Supernatant Fraction Added	[^{14}C]Isocaproate Yield (cpm/flask)	
	Expt A	Expt B
None	187 ± 15	459 (448–475)
Control	85 ± 2	184 (175–200)
ACTH	230 ± 23	288 (243–336)
3′,5′-AMP	165 ± 16	315 (289–340)

[a] In exp A: the 15,000*g* sediment contained approximately 34,000 cpm; Sephadex-treated 60,000*g* SN's were prepared from control, ACTH-stimulated, and 3′,5′-AMP-stimulated (10 μmoles/ml of incubation medium) adrenal sections; incubation conditions were otherwise as described in Tables IX and X; and the mean results plus and minus variation of duplicate incubation flasks are shown. In expt B: the 15,000*g* sediment contained approximately 89,000 cpm; dialyzed (18 hr) 0–40% ammonium sulfate fractions were prepared as in Table XI, from the 60,000*g* supernatants derived from control, ACTH-stimulated, and 3′,5′-AMP-stimulated adrenal sections; incubation conditions were otherwise as described in Tables IX and XI; the amounts (milligrams) of protein in the ammonium sulfate fractions added to control, ACTH, and 3′,5′-AMP flasks were 0.126, 0.126, and 0.133, respectively; and the mean results and the range of triplicate or quadruplicate incubation flasks in parentheses are shown.

stimulated cholesterol side-chain cleavage, and the Sephadex-treated 60,000*g* SN's derived from other rat tissues were either without effect or stimulatory. The supernatants derived from the rabbit, dog, and pig adrenal (but not the cow), on the other hand, inhibited cholesterol side-chain cleavage, suggesting a degree of organ specificity of the inhibitory factor(s).

As shown in Table XVI, the control and ACTH Sephadex-treated 60,000*g* SN's derived from incubated "normal" human adrenal cortex slices, had the same effects on cholesterol side-chain cleavage as those observed in the experiments utilizing rat SN. This was best demonstrable when human SN was incubated with labeled 15,000*g* sediment derived from rat adrenal quarters, but was also discernible with labeled sediment derived from human adrenal tumor slices. In contrast to the results with the "normal" human adrenal, the effect of the Sephadex-treated 60,000*g* SN on the cholesterol side-chain-cleaving activity obtained from incubated human adrenal tumor slices was unaffected by ACTH, and this correlated quite well with the failure of these slices to respond to ACTH, as measured by the appearance of hydrocortisone in the incubation medium.

Discussion

The present findings suggest that certain aspects of the steroidogenic effect of ACTH may be amenable to study in a cell-free system. After exposure of relatively intact adrenal tissue to ACTH *in vitro*, the steroidogenic activity of subsequently derived homogenates and subcellular fractions is enhanced, presumably *via* the accumulation of an ACTH-induced steroidogenic

TABLE XIII: Effects of Heat and Trypsin on the Cholesterol Side-Chain-Cleaving Activity of Control and ACTH Sephadex-Treated 60,000*g* Supernatant.[a]

Expt	Treatment of SN Protein Fraction	[^{14}C]Isocaproate Yield (cpm/flask)		
		15,000*g* Sediment Alone	15,000*g* Sediment + Control SN Protein Fraction	15,000*g* Sediment + ACTH SN Protein Fraction
A	None	507	177 ± 2	368 ± 26
	100° × 3 min	–	295 ±20	309 ± 7
B	Incubation at 37° for 30 min, then 50 μg of trypsin inhibitor was added	593	441 ± 44	617 ± 18
	Incubation at 37° for 30 min with 5 μg of trypsin, then 50 μg of trypsin inhibitor was added	–	518 ± 14	445 ± 30

[a] Control and ACTH Sephadex-treated 60,000*g* SN's (different supernatants in each experiment) were treated with heat or trypsin as indicated. (Trypsin alone had variable effects on the activity of the 15,000*g* sediment for [^{14}C]isocaproate formation, but soybean trypsin inhibitor alone and the combination of trypsin plus soybean trypsin inhibitor were without effect.) The 15,000*g* sediment labeled with [26-^{14}C]cholesterol contained approximately 22,000 cpm in expt A and 56,000 cpm in expt B. Other details of the incubation are provided in Tables IX and X. Where indicated, the mean value plus and minus variation of duplicate flasks is shown.

TABLE XIV: Effects of Heat on the Cholesterol Side-Chain-Cleaving Activity of Control and ACTH $(NH_4)_2SO_4$ Fractions.[a]

Expt	Treatment of $(NH_4)_2SO_4$ Fraction	[^{14}C]Isocaproate Yield (cpm/flask)		
		15,000g Sediment Alone	15,000g Sediment + Control 0–40% $(NH_4)_2SO_4$ Fraction	15,000g Sediment + ACTH 0–40% $(NH_4)_2SO_4$Fraction
A	None	505 ± 23	298 ± 24 (0.39)	439 ± 57 (0.37)
	Preincubation at 37° for 30 min	–	338 ± 33	267 ± 5
B	None	413	250 ± 10 (0.15)	–
	Preincubation at 60° for 2 min	–	404 ± 1	–

[a] Fractions obtained between 0 and 40% saturation with $(NH_4)_2SO_4$ were dialyzed for 18 hr as in Table XI, and then preincubated (or not) under the conditions described, prior to the final incubation with the labeled 15,000g sediment (31,000 and 15,000 cpm/flask in expt A and B, respectively). Other details of the incubations are provided in Tables IX and XI. In parentheses are the amounts (in milligrams) of protein in the 0–40% $(NH_4)_2SO_5$ fraction added to each incubation flask. Where indicated, the mean results plus and minus variation of duplicate incubation flasks are shown.

factor(s). This finding in itself is not surprising since Ferguson (1963) previously noted that the *in vitro* steroidogenic effect of ACTH, once established, persists in the relatively intact tissue, despite inhibition of new ACTH action by puromycin.[8] However, the finding of comparable persistence of the ACTH effect in a cell-free system permits a more direct and critical examination of the nature and function of the ACTH-induced steroidogenic factor(s).

Thus far, the major difficulty with the cell-free system is its requirement for saturating amounts of exogenous TPNH generators. This incubation condition markedly stimulates base-line steroidogenesis and consequently reduces the relative stimulatory effect of ACTH. However, the relative and absolute effects of ACTH in the cell-free system are as great as those observed when adrenal quarters are incubated with saturating levels of TPNH generators. In addition, although the relative effect is much less, the absolute increase in steroids due to ACTH in the TPNH-supplemented cell-free system is considerably greater than that occurring in unsupplemented incubations of adrenal quarters. Nevertheless, it remains uncertain whether the ACTH effects observed in the present cell-free system are fully or only partly (if at all) representative of the ACTH effects in the intact tissue.

Our present results suggest that the ACTH effect in the cell-free system is not due *directly* to ACTH or 3′,5′-AMP, or to enhanced availability of TPNH. The effect is *inducible* only in relatively intact tissue (*in vivo* as well as *in vitro*, unpublished observations) and the inductive process requires protein synthesis. The factor largely responsible for the ACTH effect is recovered in the 60,000g SN, and the ACTH effect persists, relatively unchanged, after removal of most low molecular weight substances, including steroid precursors, by Sephadex (G-25) chromatography. Relative to identically treated control SN, the Sephadex-treated 60,000g SN from ACTH-stimulated adrenals enhances total corticosteroid production when incubated with an adrenal large-particle fraction (15,000g sediment). And, when the latter has been labeled by prior incubation of adrenal tissue with [4-^{14}C]cholesterol or [26-^{14}C]cholesterol, the conversion of ^{14}C to corticosterone or isocaproic acid, respectively, is comparably enhanced. It thus seems clear that the ACTH effect on total steroid production cannot be explained simply by an accumulation of steroid precursors; rather, there appears to be an increased activity of the Sephadex-treated 60,000g SN for promoting the conversion of cholesterol (presumably mitochondrial) to pregnenolone. The conversion of pregnenolone or progesterone to corticosterone is not affected, and this agrees with previous studies in more intact systems wherein the effect of ACTH was localized to the steps between cholesterol and pregnenolone in the biosynthetic pathway (see Stone and Hechter, 1954; Karaboyas and Koritz, 1965; Billiar and Eik-Nes, 1965). It should be stressed here that substantial conversion of cholesterol to pregnenolone occurs only in the presence of mitochondria (unpublished observation with rat adrenal subcellular fractions; also see Halkerston *et al.* (1961) regarding bovine subcellular fractions); thus, although the SN itself does not effect this conversion, it apparently contains factors that control steroidogenesis by modulating this important mitochondrial function.

The inhibitory effect of Sephadex-treated 60,000g SN on the cholesterol side-chain-cleaving activity (or conversion of cholesterol to pregnenolone) of

[8] Comparable results have been obtained with chloramphenicol (unpublished observations).

TABLE XV: Specificity of Inhibitory Effects of Adrenal Sephadex-Treated 60,000*g* Supernatant on Cholesterol Side-Chain Cleavage.[a]

Addition (mg of protein)	[^{14}C]-Isocaproate Formed (cpm/flask)	% Change (*vs.* no addn)
None	742	—
Bovine serum albumin (2.0)	2817	280
Sephadex-treated 60,000*g* SN (derived from 56 mg of tissue)		
Rat adrenal (1.7)	272	−63
Dog adrenal (1.7)	558	−25
Pig adrenal (2.3)	325	−56
Cow adrenal (2.9)	1481	100
Rabbit adrenal (2.5)	169	−77
Rat liver (3.9)	150	101
Rat spleen (3.1)	812	9
Rat heart (3.4)	1119	51
Rat skeletal muscle (1.8)	1026	38
Rat testes (2.5)	844	11
Rat brain (1.5)	727	−2
Rat kidney (3.9)	1128	52
Rat thyroid (3.6)	1499	100
Rat lung (3.6)	1030	39

[a] Protein fractions were obtained after passage of the 60,000*g* SN (supernatant) through Sephadex (G-25) columns and the amount of protein derived from 56 mg of tissue is shown in parentheses. Incubation flasks contained 15,000*g* sediment labeled with [26-^{14}C]-cholesterol (approximately 80,000 cpm) derived from 56 mg of adrenal tissue, and additions as described. Other details of the incubation and experimental technique are described in Tables IX and X.

TABLE XVI: Effects of ACTH on the Cholesterol Side-Chain-Cleaving Activity of Human Adrenal 60,000*g* Supernatant.[a]

Source of Added Sephadex-Treated 60,000*g* SN	[^{14}C]Isocaproate Yield (cpm/flask)	
	I. Human Adrenal Tumor Labeled 15,000*g* Sediment	II. Rat Adrenal Labeled 15,000*g* Sediment
No SN added	798 ± 37	949 ± 20
"Normal" human adrenal slices		
1. Unincubated (2.28)	599 ± 107	113 ± 18
2. Incubated alone (2.07)	428 ± 20	607 ± 3
3. Incubated with ACTH (1.99)	588 ± 13	797 ± 1
Human adrenal tumor slices		
1. Unincubated (3.86)	539 ± 13	538 ± 52
2. Incubated alone (2.36)	566 ± 43	891 ± 14
3. Incubated with ACTH (2.57)	575 ± 32	899 ± 37

[a] The "normal" human adrenal and human adrenal tumor, obtained from patients undergoing simultaneous adrenalectomies for breast carcinoma and adrenal tumor removal, respectively, were transported to the laboratory in ice-cold saline. The adrenals were partly demedullated, the cortex was sliced with a Stadie–Riggs microtome, and three nearly equal parts of each slice were distributed to the three subgroups. Slices of subgroups 2 and 3 were preincubated for 30 min in KRB, placed in fresh KRB, and incubated for 30 min with or without ACTH (1 IU/ml; ACTH increased the level of hydrocortisone in the incubation medium from 1.73 to 2.88 μg/100 mg of tissue in the "normal" group, but had no effect on the tumor group. Labeled 15,000*g* sediment was obtained by incubating rat adrenal quarters or human adrenal tumor slices with [26-^{14}C]-cholesterol as in Table IX. The 60,000*g* SN's were prepared from the incubated or unincubated human adrenal slices, and the protein fractions were obtained after passage through Sephadex (G-25) columns. Shown in parentheses are the amounts of protein present in the fractions derived from 100 mg of tissue slices. In column I, SN protein fractions from 112 mg of human adrenal slices were incubated with labeled 15,000*g* sediment from 112 mg of human adrenal tumor slices (containing approximately 16,000 cpm). In column II, SN protein fractions from 56 mg of human adrenal slices were incubated with labeled 15,000*g* sediment from 56 mg of rat adrenal quarters (containing approximately 28,000 cpm). Other incubation conditions are described in Table IX. The mean results plus and minus variation of duplicate incubation flasks are shown.

adrenal mitochondria is noteworthy. This inhibitory effect was relatively specific for adrenal tissue extracts (but not species specific), and the stimulatory effect of ACTH (and 3′,5′-AMP) seemed to involve a partial or complete reversal of inhibition. Thus, the magnitude of the ACTH effect was usually proportional to the degree of inhibition by control SN, and the ACTH SN rarely, if ever, increased the rate of side-chain cleavage above that of sediment incubated without added SN. Although these findings suggest that ACTH decreases the amount or activity of inhibitor (thereby enhancing steroidogenesis), it is also possible that an ACTH-induced factor(s) operates independently of the control inhibitor (the latter merely lowering the base-line cleavage rate and thus increasing the relative potential effectiveness of ACTH).

The exact nature of the SN factors responsible for control inhibition and ACTH-induced stimulation

(relative to control, *i.e.*) of cholesterol side-chain cleavage is presently unknown. The results of experiments involving puromycin, $(NH_4)_2SO_4$ precipitation, dialysis, Sephadex chromatography, and lability to heat and trypsin suggest that these factors may be proteins, but more convincing studies are needed to establish this point. Hopefully, the present incubation system, employing cholesterol side-chain cleavage as the indicator (this limited reaction appeared to be affected by ACTH more consistently and in greater magnitude than net corticosterone production) will prove useful in further characterizing the nature and function of these factors.

Although the present findings support the possibility that ACTH and 3′,5′-AMP may regulate steroidogenesis *via* a protein mediator, a disconcerting finding was the nonspecific stimulation of cholesterol side-chain cleavage produced by serum albumin and extracts of extraadrenal tissues. Among other possible explanations, these nonspecific stimulatory effects may have been due to the binding of steroids by proteins with subsequent removal of feedback inhibitors of cholesterol side-chain cleavage (see Koritz and Hall, 1964). Whatever the explanation, the nonspecific stimulation does not necessarily vitiate the significance of the ACTH-induced stimulatory effect, and moreover, it underscores the significance of the inhibitory effect of control adrenal SN. Needless to say, our present knowledge is insufficient to validly assess the significance of the present findings.

References

Avigan, J., Goodman, D. S., and Steinberg, D. (1963), *J. Lipid Res. 4*, 100.

Billiar, R. B., and Eik-Nes, K. B. (1965), *Biochim. Biophys. Acta 104*, 503.

Brown, F. (1950), *Biochem. J. 47*, 598.

Bush, I. E. (1952), *Biochem. J. 50*, 370.

Butler, F. E. (1961), *Anal. Chem. 33*, 409.

Constantopoulos, G., Carpenter, A., Satoh, P. S., and Tchen, T. T. (1966), *Biochem. J. 5*, 1650.

Elliott, F. H., Birmingham, M. K., Schally, A. V., and Schonbaum, E. (1954), *Endocrinology 55*, 721.

Farese, R. V. (1964), *Biochim. Biophys. Acta 87*, 699.

Farese, R. V., and Reddy, W. J. (1963), *Endocrinology 73*, 294.

Ferguson, J. J. (1963), *J. Biol. Chem. 238*, 2754.

Garren, L. D., Ney, R. L., and Davis, W. W. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 1443.

Halkerston, I. D. K., Eichhorn, J., and Hechter, O. (1961), *J. Biol. Chem. 236*, 374.

Hawk, P. B., Oser, B. L., and Summerson, W. H., Ed. (1954), *in* Practical Physiological Chemistry, New York, N. Y., Blakiston, p 580.

Haynes, R. C., Jr., and Berthet, L. (1957), *J. Biol. Chem. 234*, 3122.

Hilf, R. (1965), *New Eng. J. Med. 273*, 798.

Ichii, S., Forchielli, E., and Dorfman, R. I. (1963), *Steroids 2*, 631.

Karaboyas, G. C., and Koritz, S. B. (1965), *Biochem. J. 4*, 462.

Koritz, S. B. (1962), *Biochim. Biophys. Acta 56*, 63.

Koritz, S. B., and Hall, P. F. (1964), *Biochemistry 3*, 1298.

Koritz, S. B., and Péron, F. G. (1959), *J. Biol. Chem. 234*, 3122.

Lantos, C. P., McNiven, N. L., Ichii, S., Bedoukian, P. Z., and Dorfman, R. I. (1964), *Steroids 3*, 43.

Lowry, O. R., Rosebrough, N. J., Farr, A. L., and Randall, R. V. (1951), *J. Biol. Chem. 193*, 265.

McKerns, K. W. (1964), *Biochim. Biophys. Acta 90*, 357.

Péron, F. G., and Robidoux, W. F. (1964), *Biochim. Biophys. Acta 82*, 125.

Porter, C. C., and Silber, R. H. (1950), *J. Biol. Chem. 207*, 925.

Roberts, S., Creange, J. E., and Young, P. L. (1965), *Biochem. Biophys. Res. Commun. 20*, 446.

Silber, R. H., Busch, R. D., and Oslapas, R. (1958), *Clin. Chem. 4*, 278.

Staple, E., Lynn, W. O., Jr., and Gurin, S. (1956), *J. Biol. Chem. 219*, 845.

Stone, D., and Hechter, O. (1954), *Arch. Biochem. Biophys. 51*, 457.