Phillips, H. J., and Andrews, R. V. (1960), *Proc. Soc. Exptl. Biol. Med. 103*, 160.
Porter, R. J. (1946), *in* Bacterial Chemistry and Physiology, New York, N. Y., Wiley, p 239.
Puck, T. T., Cieciura, S. J., and Robinson, A., Jr. (1958), *J. Exptl. Med. 108*, 945.
Pumper, R. W., Yamashiroya, H. M., and Molander, L. T. (1965), *Nature 207*, 662.
Rakowski, P. F., and Tritsch, G. L. (1966), *Exptl. Cell Res. 43*, 510.
Rejnek, J., Redmarik, E., Rerabkova, E., and Dolezal, A. (1963), *Clin. Chim. Acta 8*, 108.
Saxen, E., and Penttinen, K. (1965), *J. Natl. Cancer Inst. 35*, 67.
Spackman, D. H. (1963), *Federation Proc. 22*, 244.
Tritsch, G. L. (1966), *Exptl. Cell Res. 44*, 648.
Tritsch, G. L., Bell, J. A., and Grahl-Nielsen, G. (1967), *Exptl. Cell Res. 48*, 244.
Watson, H. E. (1908), *J. Hyg. 8*, 536.
Womack, M., and Rose, W. C. (1946), *J. Biol. Chem. 162*, 735.
Woolley, D. W. (1946), *J. Biol. Chem. 162*, 383.

Adrenal Cells in Tissue Culture. III. Effect of Adrenocorticotropin and 3′,5′-Cyclic Adenosine Monophosphate on 11β-Hydroxylase and Other Steroidogenic Enzymes*

J. Kowal†

ABSTRACT: Monolayer cultures of an adrenocorticotropin-responsive cell line from transplantable mouse adrenal tumors show an augmentation in maximum steroidogenic output in response to prolonged incubation with adrenocorticotropin and 3′,5′-cyclic adenosine monophosphate. This is associated with progressive increases in 11β hydroxylation of endogenously produced steroids. After 12–72-hr exposure to these agents, twofold or greater increases in the 11β hydroxylation of added [^{3}H]pregnenolone or [^{3}H]progesterone were found in stimulated cells. The continued presence of adrenocorticotropin was not required to elicit the stimulation. Adrenocorticotropin added *de novo* with the radioactive steroids did not stimulate the enzyme. In cells maintained in culture many months in the absence of adrenocorticotropin, as much as a tenfold stimulation could be obtained after 72-hr stimulation with adrenocorticotropin. Precise quantitation of this effect in the intact cell was obtained through the use of a sensitive radioactive assay. The properties of the enzyme in mitochondria obtained from these cultures were similar to those reported in bovine and rat adrenal systems. Reduced triphosphopyridine nucleotide was the optimum pyridine nucleotide cofactor, but Ca^{2+} (10 mM) was required to elicit the reaction. 11β Hydroxylation was also supported by pyruvate and a number of Krebs' cycle intermediates; the highest activity was obtained with isocitrate. Ca^{2+} inhibited 11β hydroxylation supported by Krebs' cycle intermediates. Cyanide was not inhibitory. Levels of stimulation comparable with that seen in the intact cells were obtained with mitochondria isolated from adrenocorticotropin-treated cells under all conditions which supported 11β hydroxylation. Adrenocorticotropin and 3′,5′cyclic adenosine monophosphate did not stimulate 3β-hydroxysteroid dehydrogenase or 20α-hydroxysteroid dehydrogenase.

The development of techniques for the maintenance of functional murine adrenal tumor cells in monolayer culture (Buonassisi *et al.*, 1962; Yasamura *et al.*, 1966) has provided the opportunity for *in vitro* studies of long-term, as well as immediate effects, of adrenocorticotropin on cell regulation. Additional advantages of this system are the ability of the cultures to maintain a basal output of steroids in the absence of adrenocorticotropin and the absence of an effect of adrenocorticotropin on cell growth. Measurable steroidogenic responses to adrenocorticotropin can be observed within 5–10 min (Kowal and Fiedler, 1968). These cells lack a 21-hydroxylase (Pierson, 1967) and possess a more active 20α-hydroxysteroid dehydrogenase than that present in mouse adrenals (Pierson, 1967). As a result, they elaborate a mixture of 20α-dihydroprogesterone[1] and 11β-hydroxy-20α-dihydroprogesterone instead of corti-

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† Faculty Research Associate of the American Cancer Society.

[1] The following trivial names have been employed: 20α-dihydroprogesterone, 20α-hydroxypregn-4-en-3-one; 11β-hydroxy-20α-dihydroprogesterone, 11β,20α-dihydroxypregn-4-en-3-one; desoxycorticosterone, 21-hydroxypregn-4-ene-3,20-dione; corticosterone, 11β,21-dihydroxypregn-4-ene-3,20-dione; preg-

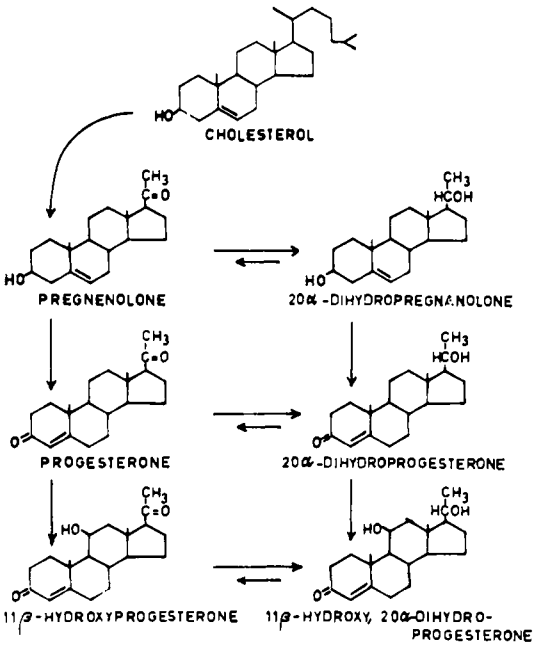

FIGURE 1.

costerone (Figure 1) (Pierson, 1967; Kowal and Fiedler, 1968).

Although the steroid output of cultures grown to confluent monolayers is quite constant, we found that repetitive stimulation with adrenocorticotropin for 24–72 hr resulted in a modest augmentation of the maximum steroidogenic response to adrenocorticotropin and to 3′,5′-cyclic AMP. Associated with this was a marked alteration in the relative proportion of the two steroid products resulting from a progressive increase in 11β hydroxylation. In this report we describe the results of our investigations of the stimulatory effects of adrenocorticotropin and of 3′,5′-cyclic AMP on the activity of 11β-hydroxylase. We have found the enzyme in these cells to be qualitatively similar to 11β-hydroxylase described in adrenal tissue from other species. The effect of continuous stimulation with both of these agents is to produce a progressive and marked increase in activity of this enzyme in the intact cell and in mitochondria isolated from stimulated cells.

Experimental Procedures[2]

Nucleotide cofactors, substrates, and enzymes were obtained from Schwarz BioResearch, Sigma Biochemical, Mann Research, and Worthington Corp. Radioactive steroids were obtained from New England Nuclear Corp. and were checked for purity by silica gel thin-layer chromatography in two systems: benzene–acetone (120:30, v/v) and benzene–ethyl acetate (75:75, v/v). A stable adrenal cell line, isolated by cloning techniques (Yasamura *et al.*, 1966), was originally obtained from Dr. Gordon Sato. Cells used for these studies were grown from these stocks or were primary monolayer cultures of adrenal tumors generated in mice from these cells. Techniques of cell culture, steroid extraction, and thin-layer silica gel chromatography have been described in detail (Kowal and Fiedler, 1968).

Preparation of Carrier [^{14}C]Steroids. [^{14}C]20α-DIHYDROPROGESTERONE. Solid tumor tissue (3 g) was homogenized in 30 ml of 50 mM Tris (pH 7.4) containing 0.25 M sucrose with a Potter–Elvehjem Teflon–glass homogenizer. The supernatant (5 ml), obtained after centrifugation at 105,000*g* for 60 min, was incubated for 3 hr at 36° with 0.06 μmole (4 μCi) of [4-^{14}C]progesterone, 1 μmole of TPN$^+$, 10 Kornberg units of glucose 6-phosphate dehydrogenase, and 10 μmoles of glucose 6-phosphate in 50 mM potassium phosphate at pH 6.2. It was extracted twice with three volumes of methylene chloride, and the pooled extracts were washed twice with 0.1 N NaOH and dried. Silica gel thin-layer chromatography in benzene–acetone (120:30, v/v) revealed over 90% conversion to the 20α-hydroxysteroid. A portion of the labeled product was recrystallized with authentic 20α-dihydroprogesterone to establish radiochemical purity.

[4-^{14}C]11β-HYDROXY-20α-DIHYDROPROGESTERONE [4-^{14}C]Progesterone (5 μCi; 0.075 μmole) was added in 0.05 ml of ethanol to the medium of a single petri dish of the cloned cells. After 48-hr incubation, the medium was extracted and chromatographed in benzene–acetone (120:30, v/v). The peak corresponding to the desired product was eluted and rerun in two additional thin layer systems (benzene–ethyl acetate, 50:100, and chloroform–methanol, 95:5). No other activity appeared and an aliquot was recrystallized to constant specific activity with authentic carrier (Kowal and Fiedler 1968).

Incubations of Whole Cells with [7α-^{3}H]Pregnenolone and Assay of Products. After cultures were grown to confluent monolayers, groups of cultures were incubated with the complete medium supplemented with 10 munits/ml of adrenocorticotropin or 1.0 mM 3′,5′-cyclic AMP. A comparable group incubated in medium alone served as controls. The medium was changed every 24 hr in all cultures and the steroid content of the medium was determined. Following the incubation with adrenocorticotropin or 3′,5′cyclic AMP prescribed for each experiment, each plate of cells was washed with medium, and 2.5 ml of the medium containing [7α-^{3}H]pregnenolone or [7α-^{3}H]progesterone (0.1 or 0.05 μmole per ml and 2 × 10^6 cpm/ml) was added for periods varying from 15 min to 4 hr. In certain experiments, adrenocorticotropin was added for the first time to "control" cells at the time of the incubation with the radioactive steroid. Other additions are described separately for each experiment. The medium was extracted with methylene chloride by a previously described method (Kowal and Fiedler, 1968).

To determine the relative production of the two products, a known amount of [^{14}C]20α-dihydroproges-

nenolone, 3β-hydroxypregn-5-en-20-one; progesterone, pregn-4-ene-3,20-dione.

[2] The author is indebted to Mr. Edward Wyman, Miss Jacqueline Ludorf, and Mrs. Bertha Tyler for their excellent technical assistance. He is also grateful to Dr. L. J. Soffer for his advice and criticism.

terone (6,000–10,000 cpm) and [^{14}C]11β-hydroxy-20α-dihydroprogesterone (2000–4000 cpm) was added to an aliquot of the ^{3}H extract (200,000–400,000 cpm). The extracts were initially chromatographed in benzene–acetone (120:30, v/v). The migration of the ultraviolet absorbing compounds was monitored with a short-wave ultraviolet lamp (Mineralight). After elution of the ultraviolet-absorbing spots corresponding to the two compounds, each peak was rerun in benzene–ethyl acetate (75:75, v/v). The eluted material was treated with pyridine and acetic anhydride (Kowal and Fiedler, 1968). The acetylated compounds were rechromatographed individually in benzene–acetone (140:10, v/v), eluted again, and rechromatographed in benzene–ethyl acetate (100:50, v/v). Aliquots of the eluates were counted after each chromatographic step to determine the ratio of the two isotopes. After our initial experience, we found that a single thin-layer chromatogram in benzene–acetone followed by acetylation and rechromatography in a less polar benzene–acetone system sufficed to purify each compound (see Results).

Fractionation of Cells. After removing the medium, the cells were washed twice with saline and removed with a rubber policeman. Each group of cells was pooled from five (100 × 15 mm) or ten (60 × 10 mm) petri dishes (approximately 3–5 × 10^{7} cells). The cells were sedimented by a brief centrifugation and they were washed once with "RSB" buffer of Penman (1966) (10 mM Tris, pH 7.4, 10 mM KCl, and 0.5 mM EDTA). After removal of the buffer, they were resuspended in 3 ml of RSB and were allowed to swell for 10 min in ice. They were homogenized with 10–15 strokes of a tight-fitting all-glass Dounce homogenizer. The homogenate was centrifuged at 700*g* for 10 min in an International PR-2 centrifuge at 4°. The precipitate was rehomogenized in 2 ml of RSB, the centrifugation was repeated, and the supernatants were pooled. The complete removal of nuclei was routinely checked by microscopy. This supernatant was centrifuged at "10,000*g*" for 15 min in an International B-20 centrifuge. The 10,000*g* pellet was washed twice and resuspended in the same buffer supplemented with 0.25 M sucrose. The "10,000*g*" supernatant was separated into "microsomal" and "soluble" fractions by centrifugation at 105,000*g* for 60 min in a Beckman L-2 ultracentrifuge. The protein concentration in each fraction was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Assay of 11β-Hydroxylase Activity in Homogenates. For most experiments, the basic assay mixture contained, in 1 ml of 50 mM Tris (pH 7.4), 5 mM $MgSO_4$, 0.1% bovine serum albumin, and 0.6 mM desoxycorticosterone. Other additions to the incubation mixture are cited under each experiment. Incubations were carried out in conical extraction tubes in a Dubnoff shaking incubator for 15–45 min. The reaction was stopped by the addition of 5 ml of methylene chloride; the steroids were extracted, and the extracts washed with 0.1 N NaOH and water. The fluorescence of the corticosterone formed in the reaction was measured by the method of Silber *et al.* (1958). A corticosterone standard was carried throughout the incubation and extraction.

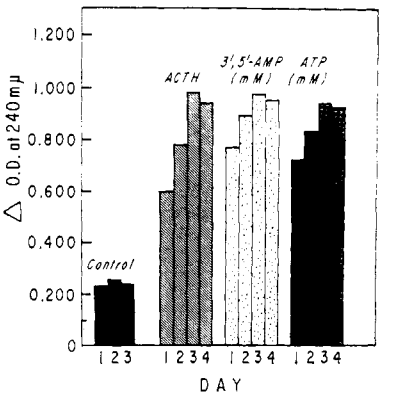

FIGURE 2: Typical steroidogenic responses of adrenal cell line to repetitive stimulation. Each bar represents the average absorbancy of identical methylene chloride extracts of medium pooled from duplicate pairs of cultures (four cultures per bar). Absorbancy was measured at 240 mμ in ethanol.

Results

Effect of Prolonged Continuous Stimulation with Adrenocorticotropin on Total Steroid Output. When experiments are performed after the cultures have grown to confluent monolayers, there is usually less than 10% difference in the total steroid output of individual plates of cells in response to either adrenocorticotropin or 3′,5′-cyclic AMP. As has been previously described, steroidogenic responses, comparable to those seen with these two compounds, can also be obtained with other adenosine nucleotides, *e.g.*, ATP, ADP, and AMP (Kowal and Fiedler, 1969).

Repetitive 24-hr stimulations with adrenocorticotropin or 3′,5′-cyclic AMP result in a greater maximum steroidogenic response in both the cloned cells maintained in long-term culture and freshly plated tumors (Figure 2). This reaches a peak by the third day of stimulation. Data obtained from experiments with cloned cells and primary cultures have shown that the greatest increases (approximately 50–75%) occur in the cloned cells. This effect is more pronounced when the response of the cells during brief periods of stimulation (1–2 hr) with adrenocorticotropin is studied following a long period of exposure to adrenocorticotropin (Table I). Thin-layer chromatography of the steroid extracts obtained during the early and late phases of stimulation showed distinct alterations in the proportion of the two major steroid products: a relative increase in 11β-hydroxy-20α-dihydroprogesterone was associated with a decreased amount of 20α-dihydroprogesterone.

This observation was subsequently confirmed by incubating a large number of cultures with medium supplemented with adrenocorticotropin or with the medium alone for five consecutive 24-hr periods. The steroid extracts from each successive 24-hr stimulation were separated by thin-layer chromatography on silica which had been washed free of ultraviolet-absorbing impurities. The ultraviolet-absorbing zone corresponding to each steroid was extracted and quantitated (Figure 3). There was a progressive increase in the 11β-hydroxylated product which reached a maximum by the third day. Cells

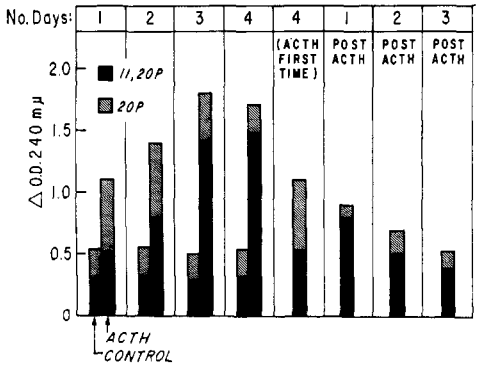

FIGURE 3: Effect of adrenocorticotropin on the endogenous production of 11β-hydroxy-20α-dihydroprogesterone and 20α-dihydroprogesterone. Ten petri dishes of cells were incubated with adrenocorticotropin (10 munits/ml) and twenty dishes served as controls. After each 24-hr period, the medium from each group was removed, and extracted with methylene chloride; the steroid extracts were dried with a stream of nitrogen and chromatographed on silica gel thin-layer plates. The plates were developed in benzene–acetone (110:40, v/v). Each ultraviolet-absorbing spot was eluted with acetone and the absorbancy of equivalent aliquots was determined. Solid bars: 20α-dihydroprogesterone; hashed bars: 11β-hydroxy-20α-dihydroprogesterone.

exposed to adrenocorticotropin for the first time on the fourth day had the same pattern as those stimulated on the first day, thus ruling out effects related to the age of the culture or the increased frequency of medium change. The altered ratio of the two products was partially reversed by removing the adrenocorticotropin and continuing the incubations for several more days. Following the 5-day period of incubation with adrenocorticotropin, there was some persistence of the adrenocorticotropin effect, the steroid output remaining above the original basal levels for several days. After trypsinization and subculture, the cells subjected to the prolonged stimulation reverted to their basal state.

Incubations of Cell Cultures with [^{3}H]Pregnenolone and [^{3}H]Progesterone. In order to study more precisely

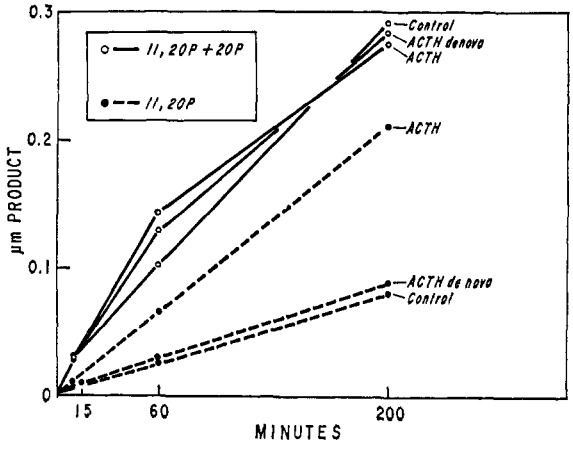

FIGURE 4: Conversion of [7α-^{3}H]pregnenolone into 11β-hydroxy-20α-dihydroprogesterone and 20α-dihydroprogesterone. Each point is the average of three cultures. The methods employed are described under Experimental Procedures.

TABLE I: Effect of Prior Incubation with Adrenocorticotropin on Short-Term Responses to Adrenocorticotropin.[a]

Prior Incubn (48 hr)	45-min Incubn (μg of 20α-hydroxysteroids/ Petri Dish)	
	Control	Adrenocorticotropin
Control	0.3	1.8
Adrenocorticotropin	0.6	3.3

[a] Six petri dishes of cells were incubated with adrenocorticotropin (10 munits/ml) for 48 hr. Six dishes served as controls. The medium was changed every 24 hr. The cultures were washed twice with medium and three cultures from each group were incubated with adrenocorticotropin (10 munits/ml) and three served as controls.

the effect of adrenocorticotropin on 11β hydroxylation as well as other enzymes in the steroidogenic pathway, experiments were performed with [^{3}H]pregnenolone containing sufficient carrier pregnenolone to saturate the 3β-hydroxysteroid dehydrogenase, 20α-hydroxysteroid dehydrogenase, and 11β-hydroxylase systems. Since the rate of conversion of pregnenolone into the products in the cells is far in excess of the endogenous rate of steroid synthesis (Pierson, 1967), little interference from endogenously synthesized steroids could be expected at the levels of pregnenolone employed. The conversion of pregnenolone and progesterone into 20α-dihydroprogesterone and 11β-hydroxy-20α-dihydroprogesterone was determined precisely by utilizing ^{14}C derivatives of these products as carriers. Following each incubation period, the steroids extracted from the medium were separated by thin-layer chromatography; each steroid product was acetylated and subjected to repeat chromatography. The yield of each product could be extrapolated from the ratio of ^{3}H products to ^{14}C carriers. A typical experiment is depicted in Table II.

Initially, cultures of cloned cells were divided into two groups, one of which was stimulated with adrenocorticotropin for 72 hr while the other received no adrenocorticotropin. At the end of this period, the medium was replaced with one containing 0.1 mM [7α-^{3}H]pregnenolone. The cultures were divided into three groups: one group which continued to receive no adrenocorticotropin, another receiving adrenocorticotropin *de novo*, and the third which had been stimulated with adrenocorticotropin for 72 hr. The kinetics of the reaction were studied by removing aliquots of medium at 0, 15, 60, and 200 min after the addition of the [^{3}H]pregnenolone (Figure 4). The formation of both products proceeded linearly. In the cells which had been stimulated for 72 hr, there was a 2.5-fold increase in the rate of 11β hydroxylation. Adrenocorticotropin added *de novo* to

TABLE II: Flow Sheet for Recovery of [^{3}H]11β-Hydroxy-20α-dihydroprogesterone from [^{3}H]Pregnenolone.[a]

	^{3}H	^{14}C	Ratio	Total ^{3}H	% Conversion
Control					
Initial extract	2,245	72.7	30.9	315,000	
Thin-layer chromatography I	1,839	376	5.01		
Thin-layer chromatography II (acetate)	1,504	389	3.99		
Thin-layer chromatography III (acetate)	1,508	394	3.94	16,400	*5.2*
Adrenocorticotropin *de novo*					
Initial extract	3,468	146	23.7	243,000	
Thin-layer chromatography I	1,001	220	4.67		
Thin-layer chromatography II (acetate)	812	233	3.61		
Thin-layer chromatography III (acetate)	815	242	3.48	14,450	*5.95*
Adrenocorticotropin 72 hr					
Initial extract	3,835	149	25.7	265,000	
Thin-layer chromatography I	5,212	375	14.7		
Thin-layer chromatography II (acetate)	5,603	399	14.2		
Thin-layer chromatography III (acetate)	5,633	404	14.1	59,700	*22.5*

[a] The steroid extracts were pooled from three cultures in each group, prior to addition of carrier. The data refer to the product yield after 60-min incubation in the experiment described in Figure 4.

control cells did not increase the synthesis of the 11β-hydroxylated product above that seen with the untreated control cells. However, the sum of the concentrations of the two steroid products was the same in all three groups. From these data, it appeared that long-term stimulation with adrenocorticotropin specifically increased 11β hydroxylation but had no effect on either the 3β-hydroxy- or 20α-hydroxydehydrogenase activity.

After 24- and 48-hr incubation with adrenocorticotropin, a stepwise increase in the rate of 11β hydroxylation could be demonstrated (Table III). Stimulated cultures incubated with the radioactive steroid in the absence of adrenocorticotropin had the same increase in hydroxylase activity as those continuing to receive adrenocorticotropin. Thus, the continued presence of adrenocorticotropin was not required to demonstrate the stimulation of 11β hydroxylation. In the experiment described, 24- and 48-hr stimulation with 1 mM 3′,5′-cyclic AMP resulted in increases in enzyme activity even greater than that seen with adrenocorticotropin (Table III). In these experiments, performed in cloned cells with levels of 11β-hydroxylase activity lower than the primary cultures, the rate of conversion of pregnenolone into 20α-dihydroprogesterone was essentially the same in all cultures. Thus no stimulation of either 3β-hydroxy- or 20α-hydroxydehydrogenase activity was effected by either agent. Once again, adrenocorticotropin added to the medium *de novo* was without effect.

Similar degrees of stimulation were seen when [^{3}H]progesterone was used in place of pregnenolone (Table III). In this experiment, a lower concentration of 3′,5′-cyclic AMP (0.3 mM) was used. In contrast to the experiment with 1.0 mM 3′,5′-cyclic AMP, there was a smaller degree of stimulation than that obtained with adrenocorticotropin, suggesting a concentration-dependent effect of 3′,5′-cyclic AMP on 11β-hydroxylase activity. Although increases in 11β hydroxylation following prolonged exposure to 3′,5′-cyclic AMP were consistently found, the increases in enzyme activity were occasionally less than that found with adrenocorticotropin.

When adrenocorticotropin and 3′,5′-cyclic AMP were added together, the degree of stimulation was the same as that found with the nucleotide alone. This was also seen in other experiments in which the response to 3′,5′-cyclic AMP was lower than to adrenocorticotropin suggesting that the nucleotide interfered with the response to adrenocorticotropin. The possible significance of this effect of 3′,5′-cyclic AMP is being investigated further. Thyroxine (0.01 mM) added to the cells simultaneously with adrenocorticotropin during the preincubation was found to enhance the stimulatory effect of adrenocorticotropin on 11β hydroxylation, but did not enhance the steroidogenic response during the actual periods of stimulation with adrenocorticotropin. In an additional experiment thyroxine added to the cells alone was found to have no stimulatory effect on either steroidogenesis or 11β hydroxylation. Estradiol was also added to the cultures with and without adrenocorticotropin and found to have no effect on either the endogenous rate of steroidogenesis or the stimulation of 11β hydroxylation. The possibility that the increase in 11β hydroxylation was due to the higher concentration of steroids resulting from adrenocorticotropin stimulation was ruled out since the addition of progesterone or 20α-dihydroprogesterone to the medium in concentrations comparable with that achieved during long periods of adrenocorticotropin stimulation had no stimulatory effect on 11β-hydroxylase activity (Table III).

To determine whether these findings may have re-

TABLE III: Incubation of "Lowest Activity" Cloned Cells with [7α-^{3}H]Pregnenolone and [7α-^{3}H]Progesterone.[a]

	mμmoles of 11,20-Progesterone	mμmoles of 20-Progesterone
I. [7α-^{3}H]Pregnenolone[d]		
Control	3.0	145.6
Control	3.1	140.0
Control[b]	3.8	141.8
Adrenocorticotropin, 24 hr	6.0	149.4
Adrenocorticotropin, 48 hr	14.2	137.3
Adrenocorticotropin, 48 hr[d]	13.3	135.4
3′,5′-AMP, 24 hr, 1 mM	13.3	154.4
3′,5′-AMP, 48 hr, 1 mM	26.6	140.2
3′,5′-AMP, 48 hr, 1 mM[b]	26.6	141.0
Progesterone, 48 hr	3.2	142.6
II. [7α-^{3}H]Progesterone[c]		
Control	3.8	132.0
Control	3.8	125.3
Adrenocorticotropin, 72 hr	12.6	121.2
Adrenocorticotropin, 72 hr	13.4	123.6
3′,5′-AMP, 0.3 mM, 72 hr	7.0	128.7
3′,5′-AMP, 0.3 mM, 72 hr	6.3	121.8
Adrenocorticotropin and 3′,5′-AMP, 0.3 mM, 72 hr	5.7	130.7
Adrenocorticotropin and 3′,5′-AMP, 0.3 mM, 72 hr	7.1	124.1
Adrenocorticotropin and thyroxine, 0.01 mM, 72 hr	17.1	113.5
Adrenocorticotropin and thyroxine, 0.01 mM, 72 hr	16.6	118.7

[a] Product yield after 3.5-hr incubation with 300 mμmoles of [7α-^{3}H]pregnenolone/culture. Each number is averaged from pooled duplicate cultures. [b] Adrenocorticotropin included in incubation with radioactive steroid. [c] Incubation of cloned cells with [7α-^{3}H]progesterone (150 mμmoles/culture). [d] Adrenocorticotropin or 3′,5′-AMP omitted from incubation with radioactive steroid.

TABLE IV: Incubation of Freshly Plated Tumor Cells with [7α-^{3}H]Pregnenolone or [7α-^{3}H]Progesterone.[a]

	Substrate	% 11,20-Progesterone	% 20-Progesterone
T916B control	Pregnenolone	0.86	5.7
T916B adrenocorticotropin	Pregnenolone	1.62	5.9
T916B control	Progesterone	1.83	20.6
T916B adrenocorticotropin	Progesterone	3.64	16.6
T919 control	Pregnenolone	0.79	4.7
T919 adrenocorticotropin	Pregnenolone	1.56	5.9
T919 control	Progesterone	1.49	13.6
T919 adrenocorticotropin	Progesterone	4.79	20.8

[a] Cells were incubated with adrenocorticotropin for 40 hr prior to the onset of the experiment. Incubation with the radioactive steroid (150 mμmoles) was carried out for 100 min. The data were obtained from extracts pooled from three cultures in each group.

sulted from an artifact arising from the conditions of long-term culture, primary cultures from several tumor lines with higher basal levels of 11β-hydroxylase activity were stimulated with adrenocorticotropin. After 40 hr, two- to threefold increases in 11β hydroxylation were observed (Table IV). A significant stimulation of 11β hydroxylation was always obtained in cells which manifested good steroidogenic responses to adrenocorticotropin or 3′,5′-cyclic AMP. In the shorter term incubation used in this experiment (100 min), the syn-

thesis of 11β-hydroxy-20α-dihydroprogesterone proceeded at a higher rate from progesterone than from pregnenolone (Table IV). This is essentially what one would expect since one less enzyme step, which may be rate limiting, is involved.

In all the experiments described, additional peaks of radioactivity corresponding to 20α-dihydropregnenolone and 11β-hydroxy-20α-dihydropregnenolone were seen (Kowal and Fiedler, 1968), but in the longer incubations (>3 hr), these compounds were present in relatively insignificant amounts.

11β Hydroxylation in Isolated Mitochondria. In order to determine whether the increase in 11β-hydroxylase activity represented an actual increase in enzyme activity or may have resulted secondarily from such factors as increased mitochondrial permeability, facilitation of the intracellular transport of steroids, alterations in the concentration of cofactors, etc., additional experiments were performed with cell-free extracts of cells which had been stimulated with adrenocorticotropin.

Mitochondria from these cells could hydroxylate desoxycorticosterone to corticosterone, which could be measured fluorometrically (Silber *et al.*, 1958). Since certain 20α-hydroxysteroids fluoresce in ethanolic sulfuric acid, the specificity of this assay for corticosterone was tested by incubating the 100,000*g* supernatant, which contains the 20α-hydroxysteroid dehydrogenase activity, with TPNH and desoxycorticosterone. This resulted in no increase in fluorescence above blank levels. Similarly, corticosterone was incubated with mitochondria and supernatant in all of the experiments to be described; the fluorescence of the steroid was not altered.

Mitochondria were prepared by several techniques to determine optimum conditions for 11β hydroxylation. Cells were either homogenized in 0.25 M sucrose–0.05 M Tris with a Teflon–glass Potter homogenizer, treated with Nagase according to the method of Kobayashi *et al.* (1966), or swelled in hypotonic buffer and homogenized in a Dounce homogenizer according to the method described by Penman (1966). Since no significant differences in activity were obtained, the Penman procedure was used with one modification. After the initial separation in hypotonic buffer, mitochondria were resuspended in 0.25 M sucrose in the remaining purification steps.

The properties of the enzyme were initially determined in "control" cells from a primary culture with relatively high 11β-hydroxylase activity (Table V). Essentially no activity could be seen with TPNH in the absence of Ca^{2+}. The highest activity was obtained with 10 mM Ca^{2+}. These results are similar to those reported by Peron in normal rat adrenal mitochondria (Peron, 1964). This effect has been attributed to the swelling of mitochondria by this ion, the swollen mitochondria presumably becoming permeable to the exogenous TPNH. The level of activity was further augmented by the addition of 5 mM Mg^{2+}, while ATP reversed the stimulatory effects of Ca^{2+}. In mitochondria of other systems, ATP is known to inhibit the swelling induced by Ca^{2+} (Peron *et al.*, 1966). In contrast, when 11β hydroxylation was supported by isocitrate and Mg^{2+}, Ca^{2+}

TABLE V: 11β Hydroxylation in Mitochondria Isolated from "High-Activity" Control Cells.[a]

Addition to Incubation (mM)	mμmoles of Corticosterone Produced/mg of Protein
I.[b] TPNH (0.2)	0.3
TPNH + EDTA (1)	0.2
TPNH + Mg^{2+} (5)	0.4
TPNH + Ca^{2+} (10)	16.8
TPNH + Mg^{2+} + Ca^{2+}	21.4
TPNH + Ca^{2+} + ATP (2)	2.7
TPNH + ATP	0.3
Isocitrate (10) + Mg^{2+} (5)	14.3
Isocitrate + Mg^{2+} + Ca^{2+} (10)	0.6
Isocitrate + Mg^{2+} + ATP (2)	15.0
Succinate (10)	2.4
Malate (10)	2.0
II.[c] TPNH + Mg^{2+} + Ca^{2+}	13.6
TPNH + Mg^{2+} + Ca^{2+} + albumin (1 mg/ml)	16.2
Isocitrate + Mg^{2+}	4.7
Isocitrate + Mg^{2+} + albumin	11.8

[a] The basic incubation mixture contained, in 1.0 ml of 50 mM Tris (pH 7.4), 60 mμmoles of desoxycorticosterone and 0.3 mg of mitochondrial protein. Incubation was carried out for 20 min at 36° in air. [b] Experiments using "mitochondrial" fraction from freshly homogenized cells. [c] The same mitochondria after storage for 72 hr at 0° in Tris 50 mM (pH 7.4) containing 0.25 M sucrose.

blocked the enzyme activity and ATP had no effect on the isocitrate supported reaction. In this experiment, succinate and malate supported the reaction but the rate was only 10–20% of the TPNH supported activity. Bovine serum albumin had no effect on the reaction rate. However, if the mitochondria were allowed to stand for 72 hr at 0°, approximately 40% of the TPNH–Mg^{2+}–Ca^{2+} and 80% of the isocitrate supported activity was lost. In such "aged" preparations, partial recovery of the TPNH activity and a threefold increase in the residual isocitrate activity could be effected by the addition of bovine serum albumin.

Table VI summarizes the relative ability of TPNH, DPNH, pyruvate, and a variety of Krebs' cycle intermediates to support this reaction. The relative rates of activity were the same in "low-activity" and "high-activity" mitochondria. DPNH, when substituted for TPNH, was able to support 11β hydroxylation at approximately one-fourth the rate of that with TPNH. The addition of TPN to the DPNH-supported reaction

TABLE VI: Relative Effect of Various Substrates and Cofactors on 11β Hydroxylation in Isolated Mitochondria.[a]

Addition to Incubation	Rel Act. (%)
TPNH–Ca^{2+}	100
DPNH–Ca^{2+}	16–29
TPN + DPNH–Ca^{2+}	85–95
Isocitrate	60–85
Isocitrate–Ca^{2+}	<5
Malate	8–15
Malate–Ca^{2+}	<3
Succinate	15–25
Succinate–Ca^{2+}	<3
Ketoglutarate	20–25
Oxalacetate	3–5
Fumarate	8–13
Citrate	3–9
Pyruvate	7–12

[a] The basic incubation mixture contained, in 1.0 ml of Tris (50 mM, pH 7.4), $MgCl_2$ (5 mM) and desoxycorticosterone (60 mμmoles). The concentration of $CaCl_2$, pyruvate, and the Kreb's cycle intermediates was 10 mM. The concentration of TPNH, TPN, or DPNH was 0.2 mM. Data are pooled from five different experiments utilizing high- and low-activity cell lines and represent the entire range of activity found.

TABLE VII: 11β Hydroxylation in Mitochondria from Control and Adrenocorticotropin-Stimulated Cells.[a]

Addition	mμmoles of Corticosterone Formed/mg of Protein	
	Control	Adrenocorticotropin
Mitochondria		
TPNH	<0.5	1.0
TPNH, Mg^{2+}, Ca^{2+}	5.5	33.5
TPNH, Mg^{2+}, Ca^{2+}, KCN (1 mM)	3.5	30.0
TPNH, thyroxine (0.1 mM)	<0.5	1.0
Isocitrate, Mg^{2+}	3.5	22.5
Isocitrate, Mg^{2+}, thyroxine	2.5	25.5
Isocitrate, KCN	2.0	20.0
Supernatant		
TPNH, Ca^{2+}, Mg^{2+}	<0.5	<0.5
Isocitrate, Mg^{2+}	<0.5	<0.5
Supernatant + mitochondria		
TPNH, Ca^{2+}, Mg^{2+}	7.0	54.4
Isocitrate, Mg^{2+}	7.5	32.5

[a] Cloned cells were stimulated with adrenocorticotropin for 72 hr. Incubation conditions were the same as in Table V.

resulted in almost complete return to full activity, presumably due to an active transhydrogenase in the mitochondria. Isocitrate was the most effective of the Krebs' cycle intermediates, supporting the reaction at rates varying from 60 to 85% of the TPNH activity. Although the reaction could be supported by oxalacetate and citrate, the reaction rates with these two compounds were very low. Slightly higher rates were seen with pyruvate, fumarate, and malate while succinate and α-ketoglutarate were approximately twice as effective as the latter compounds. The addition of Ca^{2+} to the incubation mixture containing any of the Krebs' cycle intermediates abolished the activity. These data are qualitatively similar to those of Peron *et al.* (1966) (rat adrenal) and Grant and Brownie (1955) (bovine adrenal).

Adrenocorticotropin produced an increased level of enzyme activity under all conditions employed (Table VII). When the activity was tested in the presence of TPNH and Ca^{2+}, mitochondria obtained from cloned cells which had been stimulated for 72 hr with adrenocorticotropin had a sixfold higher rate of enzyme activity than mitochondria obtained from unstimulated cells. When mitochondria from both groups of cells were studied in the absence of Ca^{2+}, no activity was seen in either group. No effect was obtained when thyroxine was substituted for Ca^{2+} in the incubation. A comparable degree of stimulation was also observed when isocitrate was substituted for TPNH and Ca^{2+}. A slight but probably nonsignificant enhancement of the isocitrate supported activity was obtained when thyroxine was added. Potassium cyanide added in a concentration sufficient to block cytochrome oxidase activity inhibited both the TPNH and isocitrate supported reactions by less than 10%. No measurable 11β-hydroxylase activity was found in the 100,000*g* supernatant fraction in both control and stimulated cells. However, in both control and stimulated cells, the addition of the supernatant to mitochondria resulted in a 60% augmentation in the rate of 11β hydroxylation in the presence of TPNH and Ca^{2+} and by approximately 50% in the presence of isocitrate. The supernatant added alone to mitochondria was inactive in supporting 11β hydroxylation in the presence or absence of TPNH (without Ca^{2+}). Since the enhancement of activity by the supernatant occurred both in control and adrenocorticotropin stimulated mitochondria, this suggested that the effect of adrenocorticotropin was primarily on the mitochondrial enzyme system and not on any additional factors present in the cytosol.

Data pooled from six additional experiments showed that stimulation with adrenocorticotropin for 48–72 hr resulted in 3–10-fold increases in 11β-hydroxylase activity in mitochondria isolated immediately after terminating the incubation. This occurred in high-activity

TABLE VIII: 11β Hydroxylation in Mitochondria Obtained from Control and Adrenocorticotropin-Stimulated Cells using 20α-Dihydroprogesterone as Substrate.[a]

	Recovery at End of Incubation (%)			11,20-Progesterone/(11,20-Progesterone + 20-Progesterone)
	11,20-Progesterone	20-Progesterone	Progesterone	
TPNH–control	0.14	41.9	58.3	0.003
TPNH–adrenocorticotropin	0.28	28.0	72.4	0.010
TPNH + Ca^{2+}–control	1.6	25.2		0.060
TPNH + Ca^{2+}–adrenocorticotropin	3.8	25.1	75.9	0.151
Isocitrate–control	3.0	64.0	34.2	0.045
Isocitrate–adrenocorticotropin	7.9	49.3	31.8	0.138
Succinate–control	0.8	69.8	31.3	0.011
Succinate–adrenocorticotropin	2.7	57.3	38.5	0.045
No addition–control	0	45.0	44.0	0
No addition–adrenocorticotropin	0	52.9	49.1	0

[a] Incubation conditions are the same as described in Table V, except that 60 mμmoles of [7α-^{3}H]20α-dihydroprogesterone was used as substrate. [^{14}C]Progesterone, [^{14}C]20α-dihydroprogesterone, and [^{14}C]11β-hydroxy-20α-dihydroprogesterone were added to the radioactive steroid extract as carriers. 11,20-Progesterone:11β-hydroxy-20α-dihydroprogesterone, 20-progesterone:20α-dihydroprogesterone.

cell lines as well as the low-activity cloned lines. Of interest was the consistent finding that the cloned cells, which had the lowest basal level of 11β hydroxylation and which had been out of the animal for the longest time, had the greatest responses to adrenocorticotropin.

The higher levels of activity of 11β hydroxylation in mitochondria from stimulated cells could also be demonstrated with 20α-dihydroprogesterone as substrate. Experiments with this steroid were complicated by the reoxidation of the 20α-hydroxyl group to the 20 ketone during the incubation, particularly when TPNH was used as a cofactor. Smaller degrees of reoxidation occurred when succinate or isocitrate were used. Reoxidation of the 20α-hydroxyl group could occur when the substrate was added to mitochondria alone. On Table VIII, the data are shown in a typical experiment in which [^{14}C]11β,20α-dihydroprogesterone, [^{14}C]20α-dihydroprogesterone, and [^{14}C]progesterone were added as recovery standards. The results were qualitatively similar to those already cited.

Discussion

The possibility that the steroidogenic response to adrenocorticotropin and 3′,5′-cyclic-AMP could be attributed to alterations in the activity of one or more enzymes involved in steroid metabolism has been the subject of investigation in a number of laboratories (Hilf, 1965; Roberts *et al.*, 1965). The approach to the problem has been hampered by the small and variable increases in activity demonstrated under the relatively short-term conditions employed *in vitro*. *In vivo*, the growth promoting effects of adrenocorticotropin are difficult, if not impossible, to separate from the steroidogenic effects. Thus, the significance of data obtained from the study of individual enzymes taken out of contest of the cellular environment are open to speculation.

The tissue culture preparations used for these studies have the advantage of providing a relatively stable *in vitro* preparation which replicates and can be maintained without added adrenocorticotropin in the growth medium. The amount of adrenocorticotropin present in the medium from the added horse and fetal calf sera is less than 5 pg/ml (Kowal and Fiedler, 1968), thus ruling out any significant contribution from this source. Our early studies with a pure strain of cells which had been maintained for more than 1 year in continuous culture showed that highly reproducible responses to adrenocorticotropin could be obtained in each generation after repeated subculturing. However, repetitive stimulation with adrenocorticotropin led to variable increases in the maximum steroidogenic response. This was particularly pronounced when cells were stimulated for short periods (1–2 hr) following longer periods of prior stimulation. This was not merely an adaptive response to the long-term culture conditions, since freshly plated tumor cells, which had higher levels of steroidogenic activity, also manifested augmented responses to repetitive stimulation with adrenocorticotropin. Thus, there appeared to be a dual effect of adrenocorticotropin, the immediate and continuous stimulation of steroidogenesis and an enhancement of the capacity of the cells for steroid secretion. Associated with this latter effect were marked increases in the rate of 11β hydroxylation. These initial findings pointed to a clear-cut stimulatory effect on an enzyme in the steroidogenic pathway

not implicated in the acute response to adrenocorticotropin.

Since the early work of Grant and Brownie (1955) and Sweat and Lipscomb (1955), in which they demonstrated the mitochondrial localization of this enzyme system in bovine adrenal tissue, the enzyme system has been extensively characterized in adrenal tissue from a variety of species. They showed its dependence upon TPNH as a cofactor, and suggested that the action of various Krebs' cycle intermediates as substrates involve the generation of intramitochondrial TPNH for the hydroxylase reaction. Peron has published data on utilization of Krebs' cycle intermediates and nucleotide cofactors in mitochondria from rat adrenals (Guerra *et al.*, 1966). He found that the action of pyridine nucleotides in cell-free mitochondrial preparations required the addition of Ca^{2+} ions, presumably to induce swelling of the mitochondria with a resultant increase in their permeability for exogenous TPNH. Since the mechanisms by which isocitrate, succinate, and other Krebs' cycle intermediates support the reaction appear to involve the stimulation of endogenous mitochondrial TPNH, Ca^{2+} was not required. In fact, 11β hydroxylation supported by these compounds was inhibited with Ca^{2+}. Simpson and Estabrook (1968) have recently proposed a model for 11β hydroxylation in bovine adrenal mitochondria which suggests a dependence on malic enzyme for the generation of TPNH. The 11β-hydroxylase in intact mitochondria from our tissue cultures is qualitatively similar to the other reported systems. The major differences appear to be in the quantitative effects of various Krebs' cycle intermediates on enzyme activity. Optimum activity is obtained with TPNH only in the presence of Ca^{2+}. As in the rat adrenal, isocitrate was the most effective of the Krebs' cycle intermediates, while succinate, citrate, and α-ketoglutarate were poorer substrates in our system than in the rat adrenal. Similarly, malate which is very effective in bovine adrenal mitochondria proved to be a poor substrate in our system. The existence of an active transhydrogenase was confirmed by experiments utilizing DPNH and TPN. Similar to the findings of Peron *et al.* (1966) with the rat adrenal, the 100,000*g* supernatant augmented 11β hydroxylation in washed mitochondria when the reaction was supported by either TPNH or isocitrate. However, in our experiments, the supernatant could not support hydroxylation in the absence of either of these agents.

Demonstrable stimulation of 11β hydroxylation by adrenocorticotropin in the intact cell required at least 12-hr incubation. The relative specificity of this effect was suggested by the absence of any stimulation of 3β-hydroxydehydrogenase or the 20α-hydroxydehydrogenase activities in the cell. Since the stimulation could be demonstrated in mitochondria obtained from cells treated with adrenocorticotropin under all the experimental conditions employed, it seems likely that the activation represents a direct effect on the enzyme system rather than secondary effects related to alterations in the compartmental structure of the cell, cofactor concentrations, or permeability of the cell to steroids. The finding of comparable degrees of elevated enzyme activity in mitochondria with TPNH or a variety of Krebs' cycle intermediates suggested that this activation might involve the final common pathway of 11β hydroxylation perhaps in the P_{450} cytochrome itself. The inhibition of cytochrome oxidase activity by potassium cyanide had no effect on 11β hydroxylation in either the control or stimulated mitochondria.

In experiments performed in the laboratory of Dr. R. W. Estabrook, we have demonstrated the presence of a P_{450}-cytochrome in mitochondria of "high-activity" primary cultures. However, in the cloned cells, we have been unable to detect it. Since the millimolar extinction coefficient of the P_{450} of bovine adrenal mitochondria is approximately 100 and the turnover is 10–20 molecules of desoxycorticosterone/molecule of P_{450}, the levels of enzyme activity in these cells are under the limit of sensitivity of the assay. The biochemical similarity of the enzyme systems involved in the initial 20α hydroxylation of cholesterol to 11β-hydroxylase (Wilson and Harding, 1968) suggests the possibility that similar mechanisms might be involved in the augmented steroidogenic response as well.

In all experiments, 3′,5′-cyclic AMP mimicked the effect of adrenocorticotropin. However, high concentrations of the nucleotide were required to obtain both the steroidogenic and stimulatory effects on 11β-hydroxylase. This finding has been common to all adrenal tissues studied. Although experiments were also performed with ATP and AMP, two other nucleotides found to have steroidogenic activity in these cells, their effect on 11β hydroxylation was too variable to draw valid conclusions. The reason for the enhancement of the effect of adrenocorticotropin by thyroxine in the intact cell remains conjectural, since we failed to see any effect of thyroxine on steroidogenesis in the intact cell or on 11β hydroxylation in cell-free preparations.

The monolayer cultures exposed to adrenocorticotropin adapt from resting cells with relatively low steroidogenic activity to cells which maintain a continuously high output of steroid hormones. This is associated with a selective increase in the activity of certain key enzymes, examples of which are the mitochondrial steroid hydroxylases. Of interest was the finding that long-term culture results in a decline in 11β-hydroxylase activity, whereas primary cultures of tumors generated in the animal from these cells have higher levels of activity. This would suggest that the capability for synthesis of the enzyme during cell replication in culture is diminished or its rate of degradation increased. Since the stimulatory effects of adrenocorticotropin are greatest in these cells, it would be reasonable to assume that adrenocorticotropin may be operative at a level involving the regulation of synthesis or degradation of specific proteins. The elucidation of the mechanism of this effect awaits further study.

Additional experiments have been performed which rule out the possibility that the effect of adrenocorticotropin on 11β hydroxylation may be representative of a more general stimulation of mitochondrial enzyme activity, an increase in cytochrome levels, or is due to an increased net synthesis of mitochondrial protein. These data will be presented in a forthcoming publication.

References

Buonassisi, V., Sato, G., and Cohen, A. I. (1962), *Proc. Natl. Acad. Sci. U. S. 48*, 1184.
Grant, J. K., and Brownie, A. C. (1955), *Biochim. Biophys. Acta 18*, 433.
Guerra, P., Peron, F. G., and McCarthy, J. L. (1966), *Biochim. Biophys. Acta 117*, 433.
Hilf, R. (1965), *N. Engl. J. Med. 273*, 798.
Kobayashi, S., Hagihara, B., Mazumi, M., and Okenuki K. (1966), *Biochim. Biophys. Acta 113*, 421.
Kowal, J., and Fiedler, R. P. (1968), *Arch. Biochem. Biophys. 128*, 406.
Kowal, J., and Fiedler, R. P. (1969), *Endocrinology* (in press).
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Penman, S. (1966), *J. Mol. Biol. 17*, 117.
Peron, F. G. (1964), *Biochim. Biophys. Acta 90*, 62.
Peron, F. G., McCarthy, J. L., and Guerra, F. (1966), *Biochim. Biophys. Acta 117*, 456.
Pierson, R. W. (1967), *Endocrinology 81*, 693.
Roberts, S., Creange, J. E., and Young, P. L. (1965), *Biochim. Biophys. Res. Commun. 20*, 446.
Silber, R. H., Busch, R. O., and Oslapas, R. (1958), *Clin. Chem. 4*, 278.
Simpson, E. R., and Estabrook, R. W. (1968), *Fed. Proc. 27*, 523.
Sweat, M. L., and Lipscomb, M. D. (1955), *J. Am. Chem. Soc. 77*, 5185.
Wilson, L. D., and Harding, B. W. (1968), *Fed. Proc. 27*, 524.
Yasamura, Y., Buonassisi, V., and Sato, G. (1966), *Cancer Res. 25*, 14.

Triplet–Singlet Energy Transfer in Proteins*

William C. Galley† and Lubert Stryer‡

ABSTRACT: Triplet–singlet energy transfer was observed in a protein–chromophore complex. The system studied was a complex of proflavin and α-chymotrypsin. The tryptophan residues of chymotrypsin were the triplet energy donors, while proflavin bound at the active site served as the singlet acceptor. The occurrence of triplet–singlet energy transfer was revealed by a delayed fluorescence from bound proflavin and selective quenching of the tryptophan phosphorescence. The over-all transfer efficiency was higher than 80%. The kinetics of the delayed proflavin fluorescence and of the residual tryptophan phosphorescence revealed that there are at least two classes of tryptophan residues and that their rate constants for triplet–singlet transfer are 20 and 2.2 sec^{-1}.

Our observations suggest that triplet–singlet transfer can serve as a useful adjunct to singlet–singlet transfer inducing proximity relationships in the 15–60-Å range in biological macromolecules. Furthermore, triplet–singlet transfer can aid in elucidating processes involving the singlet and triplet excited states of proteins.

The significant finding in this regard is that the major pathway from the excited singlet level of tryptophan residues in chymotrypsin is internal conversion rather than intersystem crossing.

Electronic excitation energy can be transferred between chromophores. Three types of transfer are known: singlet–singlet, triplet–singlet, and triplet–triplet. Singlet–singlet and triplet–singlet transfer can occur over distances of the order of 40 Å (Förster, 1959; Ermolaev and Sveshnikova, 1963), while triplet–triplet transfer requires a much closer approach of the donor and acceptor groups (Ermolaev, 1963). There are many examples of singlet–singlet transfer in proteins (Stryer, 1968). Fluorescence techniques which utilize this process have proven to be particularly useful in studying the binding of ligands to proteins (Velick, 1961; Weber and Daniel, 1966). Recently, triplet–triplet transfer has been demonstrated in an enzyme–inhibitor complex, thereby providing evidence for the presence of a tryptophan residue near the active site (Galley and Stryer, 1968). In this article, we show that triplet–singlet transfer can occur in proteins. This type of energy transfer has previously been observed in DNA–acridine dye complexes (Isenberg *et al.*, 1964).

The system studied was a complex of proflavin and α-chymotrypsin in a rigid glass at 77°K. Proflavin binds specifically to the active site of this enzyme (Glazer, 1965; Bernhard *et al.*, 1966). The tryptophan residues

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† Postdoctoral fellow of the Damon Runyon Memorial Fund. Present address: Department of Chemistry, McGill University, Montreal, P. Q., Can.

‡ Present address: Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Conn. 06520.