POLYRIBOSOME DISSOCIATION AND FORMATION IN INTACT RETICULOCYTES WITH CONSERVATION OF MESSENGER RIBONUCLEIC ACID*

By Paul A. Marks, Edward R. Burka,† Francesco M. Conconi,‡ William Perl,§ and Richard A. Rifkind**

DEPARTMENT OF MEDICINE, COLUMBIA UNIVERSITY, COLLEGE OF PHYSICIANS AND SURGEONS, AND NEW YORK UNIVERSITY RESEARCH SERVICE, GOLDWATER MEMORIAL HOSPITAL, NEW YORK

Communicated by David Shemin, April 9, 1965

It is generally recognized that protein synthesis in reticulocytes,[1-4] other mammalian cells,[5-7] plant cells,[8] and bacteria[9, 10] occurs predominately on aggregates of single ribosomes, referred to as polyribosomes. There is evidence from electron micrographs of thin sections of whole cells,[11] as well as fractions from sucrose gradient analysis of cell lysates,[11-13] to indicate that the polyribosomes are composed of two or more individual ribosomes associated by a thin continuous filamentous structure. It has been hypothesized that this filamentous structure represents a strand of messenger RNA to which ribosomes are attached and which directs the sequential addition of amino acids to the growing nascent peptide chain.

Formation and dissociation of polyribosomes occur during certain stages of cellular development. Evidence has been presented to indicate that polyribosomes appear following fertilization and increase during the early stages of embryogenesis.[14, 15] Dissociation of polyribosomes accompanies the loss in capacity for protein synthesis which characterizes erythroid cell maturation.[16-18]

In the present investigation, polyribosome dissociation and formation in intact reticulocytes has been studied by taking advantage of the fact that NaF inhibits protein synthesis in reticulocytes and causes dissociation of polyribomes.[19] Evidence is presented that during incubation of reticulocytes in the presence of NaF, polyribosome dissociation to 80S ribosomes and ribosomal subunits proceeds by simple first-order kinetics for polyribosomes of all sizes. During this polyribosome breakdown there is conservation of messenger RNA as indicated by the restoration of protein synthesis upon removal of NaF from the cells. In these cells, polyribosome formation and restoration of protein synthesis to control rates occurs without RNA synthesis. The kinetics of polyribosome formation suggest that this process is not a reversal of the process of dissociation.

Materials and Methods.—Preparation and incubation of reticulocytes: The procedures employed are similar to those described in a previous paper[1] unless otherwise noted. The reticulocytes were prepared from phenylhydrazine-treated rabbits according to the procedures of Borsook *et al.*[20] Following washing in a cold solution containing 0.15 *M* NaCl and 1.5×10^{-3} *M* $MgCl_2$, the cells were incubated in a modified Krebs-Ringer bicarbonate buffered medium.[1] C^{14}-leucine or C^{14}-valine was added in studies in which rates of protein synthesis were examined. Incubation was carried out in an atmosphere of air with agitation at the temperature and for the times described for each study. NaF was added in the concentrations indicated below. Control flasks contained comparable amounts of NaCl.

Isolation and characterization of ribosomes: The cells were lysed and the ribosomes prepared from hemolysates by procedures described in detail elsewhere.[1] Ribo-

somes where characterized by their sedimentation pattern in sucrose-density gradients.[1] The conditions of centrifugation for each experiment are indicated in the legends for the figures.

Other determinations: The rate of oxidation of 6-C^{14}-glucose or uniformly labeled C^{14}-glucose by reticulocytes was determined as described elsewhere.[21] Adenosine triphosphate concentrations of reticulocytes were determined by the method of Kornberg.[22]

Results.—Inhibition of protein synthesis by NaF: Incubation of rabbit reticulocytes with 10^{-2} *M* NaF decreases the rate of protein synthesis. This decrease is evident within 2 min, and the rate is essentially zero after 10–20 min incubation at 37° (Fig. 1). If this incubation is performed at 15°C, the decrease is evident at 10 min and the rate is essentially zero by 40–50 min. The effect of NaF is concentration-dependent. This was shown by determining the effect of NaF on the amount of protein synthesis occurring during a 30-min incubation at 37°. At 10^{-2} *M* NaF, the amount of protein synthesis was decreased 88 per cent, and at 10^{-3} *M* NaF it was decreased 69 per cent.

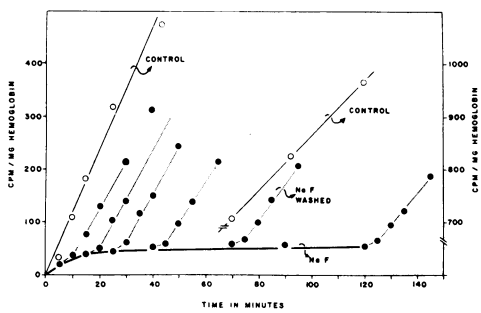

FIG. 1.—Effect of NaF on the rate of protein synthesis in reticulocytes. Reticulocytes were incubated at 37°C in the absence (open circles) or in the presence (solid line, closed circles) of NaF. At intervals, cells were removed from the NaF-containing mixture, washed, and reincubated (broken line, closed circles). See text for experimental details.

Restoration of protein synthesis in NaF-treated cells: Reticulocytes were incubated with NaF for intervals up to 120 min at 37° and then washed at 4°, twice with a solution of 0.13 *M* NaCl, 0.005 *M* $MgCl_2$, and once with the Krebs-Ringer bicarbonate solution. The cells were then reincubated at 37° in the complete medium with labeled amino acids but without NaF. Protein synthesis begins after a lag period of about 5 min if reincubation is carried out at 37°C, or after a lag period of about 20 min if reincubation is carried out at 15°C. Upon reincubation of washed cells which were preincubated with NaF, the rate of protein synthesis achieved is greater than that of control cells which were incubated for the same period of time, and is comparable to the initial rate of protein synthesis in control cells (Fig. 1).

Effect of NaF on polyribosomes: The NaF inhibition of protein synthesis in reticulocytes is associated with a progressive dissociation of the polyribosomes (Fig. 2). This was studied in detail in cells incubated at 15° rather than 37°, since polyribosome dissociation proceeded more slowly at the lower temperature, making it technically easier to obtain multiple samples during the period of change in polyribosome content. There is detectable decrease in polyribosomes after incubation for 4 min at 15°. Analysis of the patterns of sedimentation of the ribosomes in sucrose-density gradients indicates that each sedimentation class of polyribosomes decays by first-order kinetics (Fig. 3). The disappearance of polyribosomes is associated with an increase in 80S ribosomes and ribosomal subunits without appreciable loss of ribosomal material (Fig. 2). Each class of polyribosomes decreases in amount independently of the decrease in amount of any other polyribosome class.

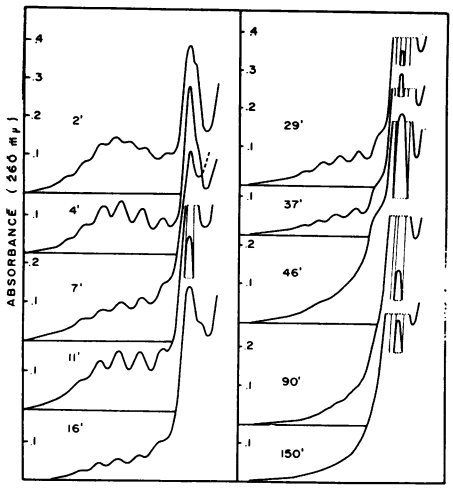

FIG. 2.—Effect of NaF on polyribosomes. Sucrose-density gradient centrifugation patterns of sedimentation of ribosomes of hemolysates prepared from reticulocytes incubated at 15°C in the presence of NaF for the time indicated. 0.5–1.0 ml of stroma-free hemolysate was layered on a 26-ml 15–30% sucrose gradient, prepared in 0.01 *M* KCl, 0.001 *M* $MgCl_2$, 0.01 *M* Tris, pH 7.4. Centrifugation was performed for 165 min at 4°C at 25,000 rpm in the SW 25.1 rotor of the Spinco ultracentrifuge, model L2. At the end of the centrifugation the gradients were collected through a flow cell (2-mm light path) of an automatic recording spectrophotometer. The broken line on graphs for 11 min and 46 min indicates 2-fold multiplication, and that for 29, 37, 90, and 150 min, 4-fold multiplication for the absorbance scale. A larger volume of lysate was layered for the 11-min gradient than for the other time points.

In addition, the rate of disappearance does not vary greatly with the size of the polyribosome. This similarity of the half-times for decay of the different classes of polyribosomes implies that the probability of dissociating a polyribosome is independent of its size, i.e., a polyribosome consisting of six ribosomes dissociates as frequently as a polyribosome consisting of five ribosomes, etc.

The disappearance of polyribosomes in reticulocytes incubated in the presence of NaF is accompanied by a loss of labeled protein from the ribosomes. This was demonstrated by studies in which the reticulocytes were incubated for 15 min at 37° in a medium containing C^{14}-leucine and then NaF, 10^{-2} *M*, was added, and the incubation continued for an additional 60 min. There was no detectable incorporation of amino acids into protein after 60 min. Polyribosome dissociation to 80S ribosomes and ribosomal subunits continued after inhibition of protein synthesis. During dissociation, the specific activity of the polyribosomes remained relatively constant. There was no accumulation of labeled protein attached to the 80S ribosomes. No detectable labeled peptides remained associated with the ribosomes when the disappearance of polyribosomes is essentially complete.

Formation of polyribosomes: Restoration of protein synthesis in cells preincubated with NaF is associated with the reappearance of polyribosomes. Polyribosomes corresponding to S values of 123 or greater are detectable within 10 min after reincubation of washed cells at 15° (Fig. 4). The proportion of ribosomes sedimenting with S values corresponding to 123S and 149S rises relatively sharply during the period between 10 and 20 min of reincubation (Fig. 5). Thereafter, the proportion of the ribosomes corresponding to 123S tends to remain relatively constant. The rate of formation of classes of polyribosomes, corresponding to S values greater than 149, is almost linear during the periods of observation. Although rates of protein synthesis comparable to that of the control are achieved within 20–30 min at 15°C, polyribosome formation comparable to that in control cells requires longer periods of incubation (90 min or longer). Extrapolation of the curves in Figure 5 back to the zero intercept suggests that there is a discrete time interval required for the formation of the larger polyribosomes. At 15° after the 173S polyribosomes appear, there is an elapse of about 10 min before any 194S polyribosomes

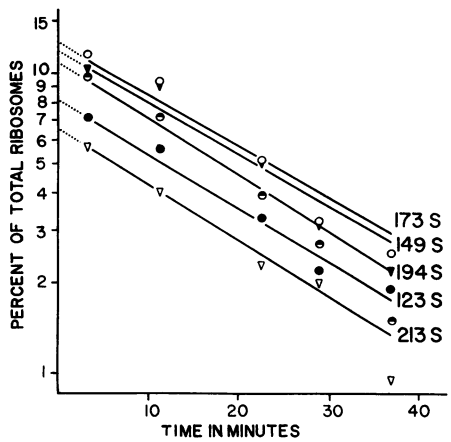

FIG. 3.—Semilogarithmic plot of the decrease in the proportion of the different classes of polyribosomes with time. These data are derived from integration of the areas under the curves of distribution of the ribosomes sedimented in sucrose-density gradients illustrated in Fig. 2. The half-times for decay of the different classes of polyribosomes are: 123S, 15 min; 149S, 15 min; 173S, 15 min; 194S, 14 min; and 213S, 13 min.

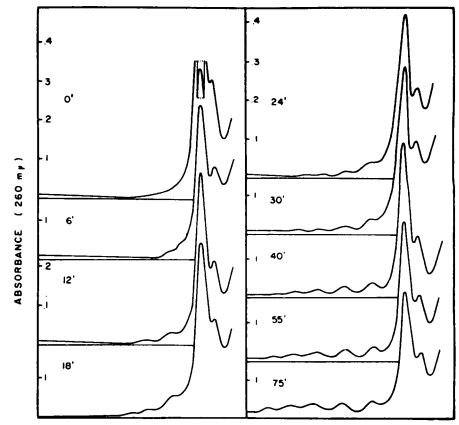

FIG. 4.—Formation of polyribosomes. Sucrose-density gradient centrifugation patterns of sedimentation of ribosomes of hemolysates prepared from reticulocytes which had been preincubated in the presence of NaF, washed to remove the NaF, and reincubated at 15° without NaF. See text for experimental details. Centrifugation and collection of the gradients was performed as indicated in the legend for Fig. 2. The broken line on the graph for zero time indicates 2-fold multiplication of the absorbance scale.

appear, and about another 15-min elapse before any 213S ribosomes appear (Fig. 5).

Polyribosome formation occurs in these reticulocytes without detectable synthesis of RNA. This is shown by studies in which the reticulocytes were preincubated for 120 min in a medium containing 10^{-2} *M* NaF. This resulted in dissociation of the polyribosomes to 80S ribosomes and ribosomal subunits as analyzed by sucrose-density gradient centrifugation of hemolysates prepared from aliquots of incubated cells. The remaining cells were washed free of NaF by the procedure indicated above, and reincubated in a complete medium free of the inhibitor to which 1 mc of P^{32} was added. Following incubation for 45 min at 37°, during which period the polyribosome distribution approached control levels, the cells were lysed and ribosomes prepared from the hemolysate. The RNA was then purified from these ribosomes and analyzed by the sucrose-density gradient centrifugation techniques for the pattern of distribution of RNA and P^{32} radioactivity.[1] There was no detectable P^{32} associated with any fraction of the RNA.

Mechanism of action of NaF: The effect of NaF on glucose oxidation, on ATP concentration, and upon rate of C^{14}-amino acid incorporation into protein and polyribosome dissociation were determined. Inhibition of oxidation of 6-C^{14}-glucose or uniformly labeled-C^{14}-glucose to $C^{14}O_2$ occurs at 30–45 min of incubation at 15° in the presence of NaF. ATP concentrations fall progressively during incubation in the presence of NaF. These data demonstrate the recognized effect of NaF in blocking glucose metabolism by inhibition of enolase activity and, as a consequence, inhibition of ATP generation.[23] It would seem reasonable to assume that inhibition of protein synthesis and polyribosome decay may be secondary to a block in the generation of high-energy phosphate compounds. This was further evalu-

ated by studying the effect of various inhibitors which are known to interfere with energy generation. Incubation of reticulocytes in the presence of 6.6×10^{-3} *M* dinitrophenol, 5×10^{-3} *M* sodium cyanide, or 6.6×10^{-3} *M* iodoacetamide caused inhibition of protein synthesis and dissociation of polyribosomes to 80S ribosomes and ribosomal subunits (Table 1). After 60 min of incubation at 37°, the degree of polyribosome breakdown observed in the presence of dinitrophenol, sodium cyanide, or iodoacetamide is similar to that which occurred in the presence of NaF.

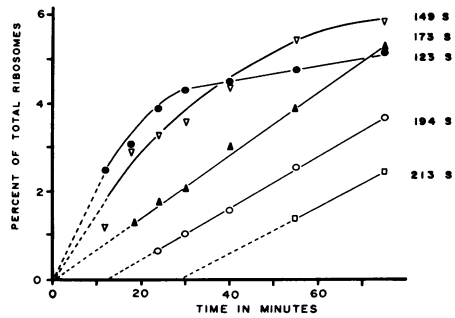

FIG. 5.—Polyribosome formation in intact reticulocytes. These data are derived from integration of the areas under the curves of distribution of ribosomes sedimented in sucrose-density gradients illustrated in Fig. 4.

Discussion.—The studies demonstrate that in intact reticulocytes NaF inhibits protein synthesis and causes a dissociation of polyribosomes to 80S ribosomes and ribosomal subunits. Polyribosomes form, and protein synthesis is restored after removal of NaF. This has provided a system which permits investigation of polyribosome structure and function in relation to protein synthesis in intact cells.

Polyribosome dissociation continues after inhibition of protein synthesis. This indicates that protein synthesis is not required for polyribosome breakdown in intact cells. This finding is in contrast with observations[24, 25] in the cell-free system which were interpreted as demonstrating a strict dependence of polyribosome breakdown on protein synthesis. During polyribosome formation, the rate of protein synthesis reaches control levels, at a time when polyribosome content is considerably less than that of control cells. This suggests that polyribosomes can vary in their efficiency in protein synthesis. This has been previously indicated by the findings of a decreased specific activity of polyribosomes as erythroid cells mature,[16, 18] and of a decrease in capacity for protein synthesis of polyribosomes of cells from subjects with thalassemia major.[26] It appears that the functional activity of messenger RNA is not simply determined by its association with ribosomes to form polyribosomes.

The process of polyribosome dissociation is not a reversal of the process of polyribosome formation. The dissociation of polyribosomes, under the present experimental conditions, proceeds by first-order kinetics. The rate of dissociation for

TABLE 1

EFFECT OF VARIOUS INHIBITORS ON PROTEIN SYNTHESIS AND POLYRIBOSOME CONTENT OF RETICULOCYTES

Addition*	Protein synthesis† (cpm/mg hemoglobin/hr)	Polyribosomes‡ (% of total ribosomes)
NaCl, 10^{-2} *M*	3200	70
NaF, 10^{-2} *M*	260	18
DNP, 6.6×10^{-3} *M*	20	10
NaCN, 5×10^{-3} *M*	50	5
IAA, 6.6×10^{-3} *M*	725	25

* Reticulocytes were incubated as described in text, with the additions as indicated. Abbreviations: DNP, 2,4-dinitrophenol; IAA, iodoacetamide.

† C^{14}-valine was employed as the labeled amino acid precursor. Incubation was for 1 hr at 37°. Other details of the procedure are indicated in the text.

‡ The proportion of total ribosomes which are polyribosomes was determined by integration of the area under the curves of the sedimentation patterns of the ribosomes in sucrose-density gradients.

polyribosomes of different size is similar. This result implies that when a polyribosome dissociates, it does not break down into other polyribosomes, but rather appears as 80S ribosomes or ribosomal subunits. These data suggest that dissociation of polyribosomes occurs as a single event which involves each ribosome in the polyribosome complex at essentially the same instant. The data do not favor a model which involves the sequential loss of single ribosomes from the polyribosome complex. Of course, sufficiently rapid sequential loss of single ribosomes is formally equivalent to a single event. The fact that NaF inhibits the formation of high-energy compounds suggests that a substance such as ATP or GTP is involved in the maintenance of the polyribosome complex. However, the mechanism by which NaF causes polyribosome dissociation is not established.

In contrast to the dissociation process, the formation of polyribosomes corresponding to sedimentation coefficients greater than 149S appears to occur as a consequence of the addition, at discrete time intervals, of single ribosomes to form the next heavier class of polyribosomes. This suggests that polyribosomes are forming by sequential addition of single ribosomes. The relatively long time (10–15 min at 15°) which elapses between the appearance of the larger classes of polyribosomes further suggests that several events may be involved in this process. The fact that the pool size of polyribosomes corresponding to 123S, and, to a lesser extent, to 149S tends to remain relatively constant throughout the period of formation of polyribosomes of heavier size would be consistent with this model, in that the rate at which these classes of polyribosomes are being formed and the rate at which they are growing to the larger polyribosomes is equal.

Polyribosome formation after NaF-induced dissociation proceeds without detectable RNA synthesis, and the rate of protein synthesis is restored to the initial control levels. These observations indicate that during polyribosome breakdown there is preservation of messenger RNA. Thus, messenger RNA can be conserved in a cell without being involved in protein synthesis. Under appropriate conditions, it can reassociate with ribosomes with resulting formation of functional polyribosome complexes. This may have an analogy in sea urchin eggs. During development of sea urchin eggs, "maternal" messenger RNA, which was presumably inactive in the unfertilized eggs, appears to account for essentially all of the synthesis of protein which occurs in the early period following fertilization.[27–29]

Summary.—Incubation of reticulocytes with NaF is associated with inhibition of protein synthesis and dissociation of polyribosomes to 80S ribosomes and ribosomal subunits with loss of nascent protein chains from the ribosomes. Removal of NaF from the cells is associated with restoration of polyribosomes and restoration of protein synthesis to control rates. The present evidence indicates that the process of polyribosome dissociation is not a reversal of the process of polyribosome formation.

* This study was supported, in part, by grants from the U.S. Public Health Service (GM 07368, CY 2332, AI 05493, HE 07482, and GM 10810) and the National Science Foundation (GB-1817).

† Trainee in hematology under grant TI-AM 5231 from the U.S. Public Health Service.

‡ Supported, in part, as the Frank J. Ficcara Fellow of The Cooleys' Anemia Foundation.

§ Address: New York University Research Service, Goldwater Memorial Hospital. The other authors are in the Department of Medicine, Columbia University, College of Physicians and Surgeons.

** Recipient of a Career Scientist Award of the Health Research Council of the City of New York under contract no. 1-336.

[1] Marks, P. A., E. R. Burka, and D. Schlessinger, these PROCEEDINGS, **48**, 2163 (1962).
[2] Warner, J. R., P. M. Knopf, and A. Rich, these PROCEEDINGS, **49**, 122 (1963).
[3] Gierer, A., *J. Mol. Biol.*, **6**, 148 (1963).
[4] Mathias, A. P., R. Williamson, H. E. Huxley, and S. Page, *J. Mol. Biol.*, **9**, 154 (1964).
[5] Wettstein, F. O., T. Staehelin, and H. Noll, *Nature*, **197**, 430 (1963).
[6] Penman, S., K. Scherrer, Y. Becker, and J. E. Darnell, these PROCEEDINGS, **49**, 654 (1963).
[7] Korner, A., and A. J. Munro, *Biochem. Biophys. Res. Commun.*, **11**, 235 (1963).
[8] Clark, M. F., R. E. F. Matthews, and R. K. Ralph, *Biochem. Biophys. Res. Commun.*, **13**, 505 (1963).
[9] Gilbert, W., *J. Mol. Biol.*, **6**, 374 (1963).
[10] Schlessinger, D., *J. Mol. Biol.*, **7**, 569 (1963).
[11] Rifkind, R. A., L. Luzatto, and P. A. Marks, these PROCEEDINGS, **52**, 1227 (1964).
[12] Warner, J. R., A. Rich, and C. E. Hall, *Science*, **138**, 1309 (1962).
[13] Slayter, H. S., J. R. Warner, A. Rich, and C. E. Hall, *J. Mol. Biol.*, **7**, 652 (1963).
[14] Monroy, A., and A. Tyler, *Arch. Biochem. Biophys.*, **103**, 431 (1963).
[15] Hultin, T., *Develop. Biol.*, **10**, 305 (1964).
[16] Marks, P. A., R. A. Rifkind, and D. Danon, these PROCEEDINGS, **50**, 336 (1963).
[17] Rifkind, R. A., D. Danon, and P. A. Marks, *J. Cell Biol.*, **22**, 599 (1964).
[18] Glowacki, E. R., and R. L. Millette, *J. Mol. Biol.*, **11**, 116 (1965).
[19] Marks, P. A., E. R. Burka, R. A. Rifkind, and D. Danon, in *Synthesis and Structure of Macromolecules*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 22 (1963), p. 599.
[20] Borsook, H., C. L. Deasy, A. J. Haagen-Smit, G. Keighley, and P. H. Lowy, *J. Biol. Chem.*, **196**, 699 (1952).
[21] Szeinberg, A., and P. A. Marks, *J. Clin. Invest.*, **40**, 914 (1961).
[22] Kornberg, A., in *Methods in Enzymology* (New York: Academic Press, 1955), vol. 2, p. 497.
[23] Lohrmann, K., and O. Meyerhoff, *Biochem. Z.*, **273**, 60 (1934).
[24] Goodman, H., and A. Rich, *Nature*, **199**, 318 (1963).
[25] Hardesty, B., J. J. Hutton, R. Arlinghaus, and R. Schweet, these PROCEEDINGS, **50**, 1078 (1963).
[26] Burka, E. R., and P. A. Marks, *Nature*, **204**, 659 (1964).
[27] Gross, P., *J. Exptl. Zool.*, **157**, 21 (1964).
[28] Maggio, R., A. Monroy, A. M. Rinaldi, and M. L. Vittorelli, *Compt. Rend.*, **260**, 1293 (1965).
[29] Spirin, A. S., and M. Nemer, in preparation.

STUDIES ON THE ROLE OF PROTEIN SYNTHESIS IN THE REGULATION OF CORTICOSTERONE PRODUCTION BY ADRENOCORTICOTROPIC HORMONE IN VIVO

BY LEONARD D. GARREN, ROBERT L. NEY, AND WARREN W. DAVIS

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, AND THE NATIONAL HEART INSTITUTE, NATIONAL INSTITUTES OF HEALTH

Communicated by C. B. Anfinsen, April 21, 1965

The primary event following the administration of adrenocorticotropic hormone (ACTH) is the immediate increase in steroid synthesis by the adrenal gland.[1] Previous studies by numerous investigators suggested that this action of ACTH was the result of specific activation of enzymes.[2, 3] However, studies showing that puromycin or chloramphenicol blocked the stimulation of corticosterone synthesis by ACTH suggested that protein synthesis was involved in this process.[4, 5] But