mass of data accumulated in this laboratory over a period of several years in support of the ribosomal pathway seems too extensive and self-consistent to be explained as the result of simple technical error or misinterpretation of experimental results. However, the fact remains that despite our best efforts, we have not been able to reproduce these experiments, and have found instead a very active enzyme system identical in all essentials with that reported by other laboratories. It is possible that this very active soluble system has masked a less active ribosomal pathway for gramicidin S biosynthesis. Experiments are in progress to investigate this possibility.

Acknowledgments

We wish to express our appreciation to Dr. Lars Nathorst-Westfelt of Aktiebolaget Astra, Soderstalje, Sweden, for his generous gift of gramicidin S.

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Biosynthesis of Adrenocorticotropin and Protein in a Cell-Free System from Bovine Anterior Pituitary Tissue*

P. Radhakantha Adiga, Prema M. Rao, Robert O. Hussa, and Theodore Winnick

ABSTRACT: A cell-free system consisting of ribosomes and pH 5 enzyme fraction, prepared from bovine anterior pituitary tissue, was found to be active in the incorporation of radioactive proline into adrenocorticotropin and mixed proteins. Following isolation by a chemical procedure, the labeled hormone was purified by paper electrophoresis.

The biosynthesis of the polypeptide resembled that of protein synthesis in its requirement for adenosine triphosphate, magnesium ion, guanosine triphosphate, and an amino acid mixture. Also both processes were inhibited by ribonuclease and by puromycin. Deoxyribonuclease and actinomycin D were noninhibitory.

In recent years there has been increasing interest in the biosynthesis of discrete proteins of known chemical constitution. Such investigations offer obvious advantages in the elucidation of basic mechanisms. Among the major types of proteins with well-defined biological activities, the polypeptide and protein hormones of vertebrates offer a number of attractions. Biosynthetic studies of this class of hormones, based on the use of surviving tissue slices, have included insulin (Wagle, 1965a; Taylor *et al.*, 1964; Humbel, 1963), thyroglobulin (Seed and Goldberg, 1963), vasopressin (Haller *et al.*, 1964), and adrenocorticotropin (ACTH)[1] (Adiga *et al.*, 1965). The first paper on the

* From the Department of Biochemistry and Biophysics, School of Medicine, University of Hawaii, Honolulu. *Received August 12, 1966.* Supported by Research grants from the U. S. Public Health Service (grant GM 09335) and the American Cancer Society.

[1] Abbreviations used: ACTH, adrenocorticotropin; ATP, adenosine triphosphate; DOC, deoxycholate; GTP, guanosine triphosphate; PEP, phosphoenolpyruvate; PK, pyruvic kinase; TCA, trichloroacetic acid.

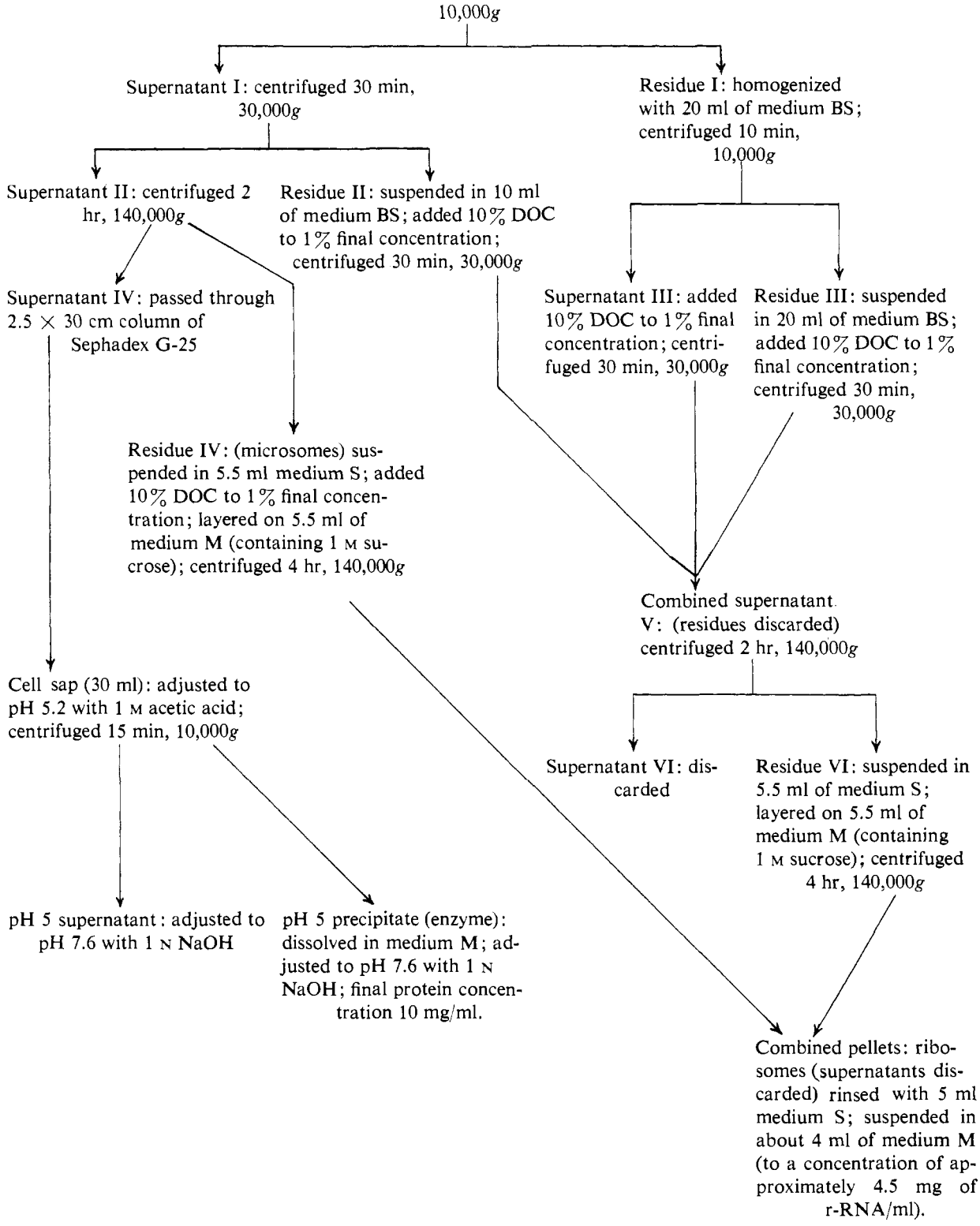

FIGURE 1: Isolation of subcellular components.

cell-free biosynthesis of a protein hormone appears to be that on insulin (Wagle, 1965b). Subsequently ribosomal systems were reported for the biosynthesis of thyroglobulin (Morais and Goldberg, 1966) and ACTH (Rao *et al.*, 1966).

The present publication describes the preparation and general properties of a ribosomal–pH 5 enzyme system from bovine adenohypophysis, which is highly

active in the incorporation of radioactive proline into ACTH and proteins. It is anticipated that this subcellular preparation will prove useful for studies of the biogenesis of the various hormones of the anterior pituitary.

Experimental Procedures

Materials. L-[3,4-^{3}H]Proline (5 c/mM) was obtained from New England Nuclear Corp. β-Mercaptoethanol, GTP, and porcine ACTH (70–115 units/mg) were purchased from Calbiochem Corp. ATP, PEP, PK, and DOC were products of Sigma Chemical Co. Bentonite (U.S.P.) from Robinson Laboratory, San Francisco, was treated according to Peterman and Pavlovec (1963), prior to use. The 20,000*g* particle size was employed in subsequent procedures. Highly purified ACTH of ovine and porcine origin were kindly supplied by Drs. C. H. Li, University of California, and A. B. Lerner, Yale University, respectively.

Preparation of Constituents of Pituitary Cell-Free System. Anterior lobes were dissected from pituitary glands of freshly slaughtered young cattle, as previously described (Adiga *et al.*, 1965). The tissue was washed with ice-cold Krebs–Ringer bicarbonate buffer, and immersed in this solution. It was subjected, with minimum delay, to the fractionation scheme of Figure 1. Usually about 9 hr were required for this entire procedure. Medium M (Munro *et al.*, 1964) consisted of 0.02 M Tris buffer (pH 7.6) containing 0.1 M KCl, 0.04 M NaCl, 0.01 M magnesium acetate, and 0.006 M mercaptoethanol. Medium S represented medium M fortified with 0.25 M sucrose, while medium BS contained, in addition, 4 mg of suspended Bentonite/ml. The final preparations of ribosomes, pH 5 enzyme precipitate, and pH 5 supernatant were generally used at once for incubation experiments. Otherwise they were stored at −20° in medium S.

It is important to point out that protein and ACTH biosynthesis were drastically reduced when the treatment with Bentonite was omitted. This effect may be due to degradative enzymes in the homogenate. The centrifugation at 30,000*g* of residues II and III and supernatant III removed remaining traces of Bentonite from these fractions.

Assay of ACTH and Protein Biosynthesis. Assays were routinely performed in duplicate, and the average values were incorporated into the tables and figures of the text. The composition of the standard reaction mixture used is shown in Table I. It was convenient to supply all of the components, except for the subcellular fractions (ribosomes and pH 5 enzyme) as 0.15 ml of a "cocktail" mixture. In some experiments, certain of the constituents were varied. In all cases, the final volume of the system was made up to 0.5 ml with medium M. The nonisotopic amino acids were employed in approximately the same relative proportions in which they occur in the ACTH molecule. The absence of other protein constituents (L-isoleucine, L-cystine, and L-threonine) in the mixture, did not appear to limit incorporation of [^{3}H]proline into protein. This latter amino acid was generally employed because of its high abundance in ACTH, as compared to protein. Earlier experiments were performed on a larger scale, with proportionally higher recoveries of radioactivity in protein and ACTH. However, the smaller 0.5-ml reaction volume was subsequently adopted to conserve biological material.

TABLE I: Standard System for Assay of Labeled Amino Acid Incorporation into ACTH and Proteins.

Component	Quantity/0.5-ml Final Vol.
Ribosomal suspension	0.1 ml (0.45 mg of r-RNA)
pH 5 enzyme	0.1 ml (1 mg of protein)
L-[^{3}H] Proline	5 μc
ATP	2.5 μmoles
GTP	0.5 μmole
PEP	1.25 μmoles
PK	6 μg
Magnesium acetate	5 μmoles
β-Mercaptoethanol	6 μmoles
Tris buffer (pH 7.6)	10 μmoles
Amino acid mixture[a]	0.025 ml

[a] This mixture contained the following amino acids (micromoles per milliliter solution): L-Ala, 0.3; L-Asp, 0.2; L-Arg, 0.3; Gly, 0.3; L-Glu, 0.5; L-His, 0.1; L-Leu, 0.1; L-Lys, 0.4; L-Met, 0.1; L-Phe, 0.3; L-Ser, 0.3; L-Try, 0.1; L-Tyr, 0.2; L-Val, 0.3.

A series of tubes, each containing 0.5 ml of incubation mixture, was incubated at 37° for 1 hr (unless otherwise indicated). The reaction was stopped by adding 0.1 ml of 50% trichloroacetic acid, followed by 1 ml of pH 5 supernatant solution (to provide carrier protein) and 1.4 ml of 50% TCA. The tubes were then left in the cold overnight. It may be mentioned that the higher concentration of TCA was used to minimize possible losses due to solubilization of ACTH (Currie and Davies, 1963) at lower TCA concentrations.

Radioactive protein and ACTH were then isolated from the precipitated material by the method used with pituitary slices (Adiga *et al.*, 1965), with the following modification. After centrifugation, the crude protein precipitates were washed four times with 4-ml quantities of 25% (instead of 5%) trichloroacetic acid containing 0.01 M L-Proline.

The chloroform (used to extract lipids from the aqueous solution of the crude ACTH) was reextracted with 0.5 ml of H_2O. The aqueous extracts were combined and evaporated to dryness *in vacuo*. The residue was extracted with 0.1 ml of 0.1 N acetic acid, and the insoluble residue was removed by centrifugation. An aliquot of the supernatant solution was used for paper electrophoresis. In this process, the solvent system of Lande *et al.* (1965) was modified to pyridine–acetic acid–H_2O (1:20:79), containing 4 M urea,

with a pH of 3.5 (instead of 5.6). The presence of urea increased the resolution of the ACTH, without markedly altering its mobility.

Following drying of the paper strips, the ACTH markers were located by staining. The radioactive strips were washed quickly with 5% TCA to remove urea. The latter substance was found to interfere with liquid scintillation counting. The labeled ACTH region on each strip was then eluted with several small portions of 50% ethanol (totaling 8 ml in volume) into counting vials, and evaporated to dryness. H_2O (0.4 ml) was added to each vial, followed by 10 ml of liquid scintillator (Hunt, 1965). Radioactivity was measured in the Packard Tri-Carb scintillation spectrometer. All assays were corrected for zero-time incubations, which were generally of the order of 1% of the standard incorporation value (approximately 2500–3000 cpm).

A typical electropherogram of labeled ACTH is shown in Figure 2. The radioactive polypeptide was not as well resolved as in tissue slice experiments. This situation may be a consequence of the present use of 25% trichloroacetic acid (instead of 5%) in the precipitation of the crude protein fraction. However, a sharp peak was invariably obtained in the region of approximately 6–10 cm, which corresponded well with the position of authentic hormone markers.

The radioactive protein from each assay, prepared as previously described, was dissolved in 0.2 ml of tetramethylammonium hydroxide, and diluted to 2 ml with ethanol. Duplicate 0.1-ml aliquots were then measured into vials containing scintillation mixture, and counted in the Model 3003 Packard Tri-Carb instrument. Zero-time radioactivities were quite low, less than 0.5% of 1-hr values.

Determination of Protein and Ribonucleic Acid (RNA). Protein was determined by the method of Lowry *et al.* (1951). Ribosomal ribonucleic acid (r-RNA) was measured as described by Scott *et al.* (1956), as modified by Fleck and Munro (1962), except that bovine serum albumin was used as carrier, instead of cell sap (Munro *et al.*, 1964).

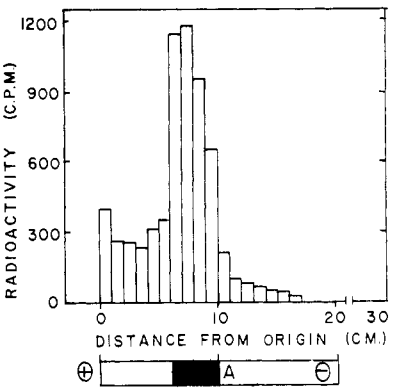

FIGURE 2: Paper electrophoresis of [^{3}H]proline-labeled ACTH, derived from the standard cell-free system. The solid region A at the bottom represents the position of both ovine and porcine ACTH standards, stained with bromophenol blue. The electrophoresis was performed for 90 min at 1200 v, using pyridine–acetic acid–water (1:20:70) of pH 3.5, containing 4 M urea.

TABLE II: Importance of Energy Sources and Amino Acid Mixture for Biosynthesis.

Constituent Omitted from Std Incubn Mix	[^{3}H]Proline Incorp (cpm)[a]	
	ACTH	Protein
Experiment 1		
None (control)	2,650	75,000
ATP	60	2,400
PEP, PK	110	4,000
ATP, PEP, PK	0	400
Experiment 2		
None (control)	2,300	74,800
GTP	1,670	28,500
Amino acid mixture	1,740	53,200

[a] The values are average results of duplicate assays.

Results

Importance of Energy Sources and Amino Acid Mixture for ACTH and Protein Biosynthesis. In Table II the standard incorporation values of 2650 and 2300 cpm into ACTH represents the utilization of approximately 0.1% of the total quantity of radioactive proline employed (2.8×10^6 cpm, representing 4 μc of ^{3}H). The 75,000 cpm recovered in the protein fraction correspond to a utilization of more than 2% of the labeled amino acid for protein synthesis. These efficiencies were found to vary considerably in experiments performed at different times, with separate batches of tissue.

It may be seen that the omission of either ATP or the ATP-generating system resulted in a severe reduction in both ACTH and protein biosynthesis. When both of these energy sources were absent, the incorporation processes were virtually abolished.

The requirements for GTP and for the amino acid mixture were less definitive. However, the omission of these components invariably led to decreases in both ACTH and protein labeling. Typical results are shown in expt 2. It is possible that the considerable degrees of biosynthesis in these cases reflect the availability of endogenous quantities of guanosine nucleotides and various amino acids, adhering to ribosomes or the pH 5 fraction.

Optimum Conditions for Biosynthesis. The incorporation of isotopic proline into ACTH and protein as a function of time is shown in Figure 3. It may be seen that the rate of ACTH labeling was rapid initially, and then decreased considerably over longer intervals

TABLE III: Importance of the Subcellular Components for ACTH and Protein Biosynthesis.[a]

Expt No.	Ribosomes (equiv to 0.9 mg of RNA)	pH 5 Enzyme Ppt (1.0 mg of protein)	pH 5 Supernatant (ml)	Original Whole Supernatant (ml)	[^{3}H]Proline Incorp (cpm)	
					ACTH	Protein
1	+	+	−	−	7,050	246,000
2	+	+	0.4	—	2,800	188,000
3	+	−	−	0.5	2,950	224,000
4	+	−	−	−	1,300	5,000
5	−	−	−	0.7	0	1,000
6	−	+	0.7	−	0	2,000
7	*b*	+	−	−	1,500	6,000

[a] The incubations were conducted at twice the standard scale (1-ml total volume). [b] Microsomes were used in place of ribosomes.

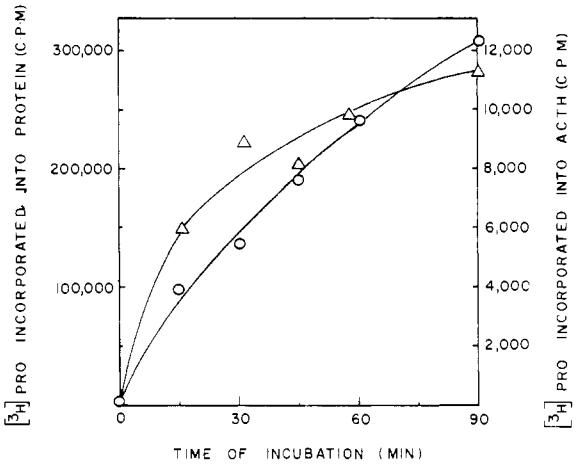

FIGURE 3: Time course of ACTH and protein biosynthesis. The incubations were conducted on twice the standard scale (1-ml reaction volume and 10 μc of [^{3}H]proline). △, ACTH; ○, protein.

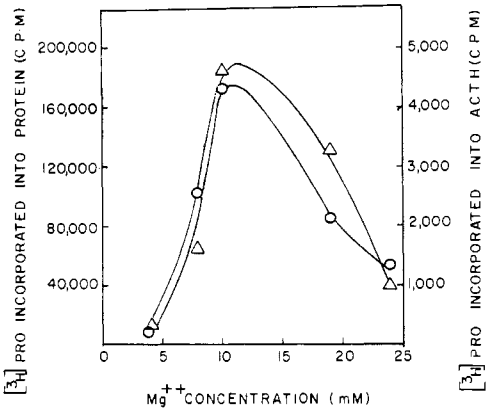

FIGURE 4: Influence of Mg^+ concentration on ACTH and protein synthesis. The incubation was conducted on twice the standard scale. △, ACTH; ○, protein.

of time. In the case of protein synthesis, the rate did not depart greatly from linearity over the 1.5-hr period of incubation.

The sensitivity of the biosynthetic processes to Mg^{2+} concentration is seen in Figure 4. The curves for both ACTH and protein synthesis appear rather similar in form, with peaks in the vicinity of 10–12 μmoles/ml.

The requirements for Na^+ (28 μmoles/ml) and K^+ (70 μmoles/ml) were found to be identical for ACTH and protein synthesis (data not given). Higher Na^+ concentrations were more inhibitory than K^+.

Role of the Subcellular Constituents. Experiment 1 in Table III shows that the incorporation of labeled proline into ACTH was highest in the standard system, containing ribosomes and pH 5 enzyme. Incorporation into protein was fairly good. When pH 5 supernatant phase was also included (expt 2), ACTH biosynthesis was reduced by more than 50%, although protein synthesis increased slightly. When original (unfractionated) supernatant was used in place of pH 5 enzyme (expt 3), a similar result was obtained. In expt 4, in which ribosomes alone were tested, almost one-fifth of the control level of ACTH biosynthesis was observed, while protein formation was greatly reduced. In the absence of ribosomes, neither the whole supernatant (expt 5), nor its component fractions (expt 6) displayed appreciable incorporative ability. Experiment 7 shows that microsomes were about 20% as active as ribosomes (in presence of pH 5 enzyme) for the incorporation of radioproline into the polypeptide hormone, although incorporation into the protein fraction was very low.

The relationship between the concentration of ribosomes and biosynthetic activity is presented in more detail in Figure 5. It may be seen that the incor-

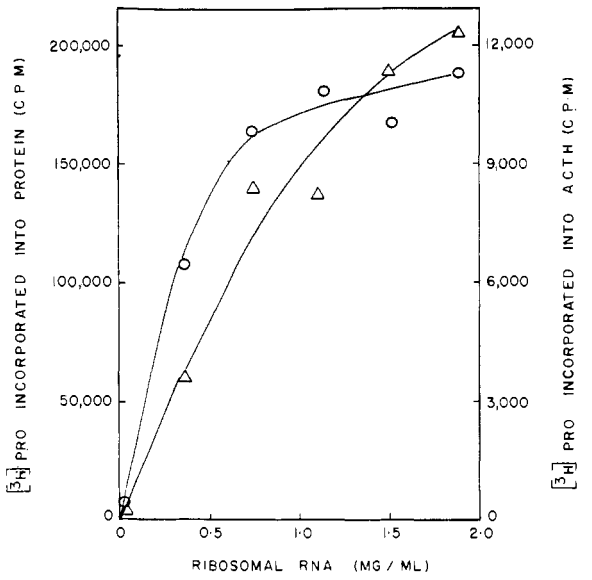

FIGURE 5: Relationship of ribosome concentration to extent of ACTH and protein biosynthesis. The incubations were conducted on twice the standard scale. △, ACTH; ○, protein.

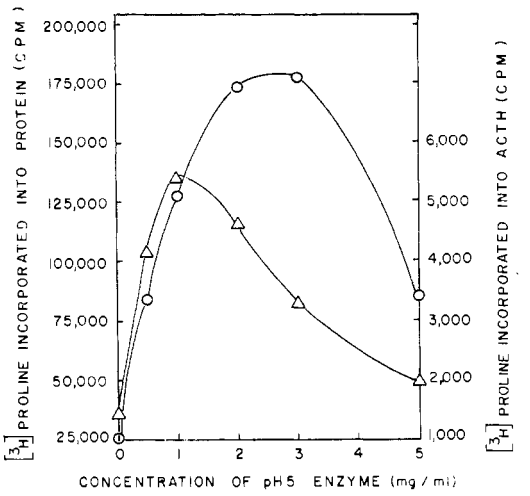

FIGURE 6: Effect of varying quantities of pH 5 enzyme on the extent of ACTH and protein synthesis. The incubation was conducted on twice the standard scale. △, ACTH; ○, protein.

poration of labeled proline into protein approached a maximum value at a level corresponding to about 2 mg of r-RNA/ml of incubation mixture. However, the curve for ACTH continued to rise in this region. It can be concluded that the concentration of ribosomes used in the standard system (approximately 1 mg of r-RNA/ml) is in the optimum region for protein synthesis, but considerably below the saturation level for ACTH formation.

It was considered desirable to also study the effect of varying the concentration of pH 5 enzyme fraction (Figure 6). Incorporation of labeled proline into ACTH was found to be optimal at 1 mg of pH 5 protein/ml. The fall in activity beyond this peak probably reflects the inhibitory effect of some supernatant phase, which may have adhered to the (unwashed) pH 5 protein. A somewhat higher pH 5 enzyme requirement, about 2–3 mg of protein/ml, was observed for maximal [^{3}H]proline incorporation into protein.

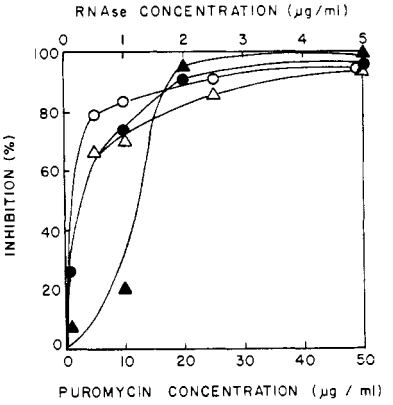

FIGURE 7: Inhibition of ACTH and protein biosynthesis by puromycin and RNAase. △, ACTH and puromycin; ▲, ACTH and RNAase; ○, protein and puromycin; ●, protein and RNAase.

TABLE IV: Stability of Ribosomes and pH 5 Enzymes on Storage for 7 Days at −20°.

System Tested	[^{3}H]Proline Incorp (cpm) Into ACTH	Into Protein
Control (fresh components)	2,800	102,000
Stored ribosomes + fresh pH 5 enzymes	1,200	63,000
Stored pH 5 enzyme + fresh ribosomes	2,650	89,000

Survival of Subcellular Components upon Low-Temperature Storage. Table IV shows that the ribosomes lost almost 60% of their activity for [^{3}H]proline incorporation into ACTH and about 40% of protein-labeling ability after storage for 1 week at low temperature. The pH 5 fraction was more stable, and retained approximately 95% of its biosynthetic activity for ACTH and close to 90% for protein.

Inhibitors of Protein Synthesis. Two types of evidence, which support the view that ACTH and general protein synthesis have similar mechanisms, are presented in Figure 7. Both biosynthetic processes were sensitive to puromycin, and were almost completely blocked

at higher concentrations of this classic inhibitor. In the ribonuclease (RNAase) experiment, incorporation of [^{3}H]proline into ACTH was less affected by low concentrations of the enzyme, than was labeling of protein. However, about 90% inhibition of both syntheses resulted at 2 μg of RNAase/ml, and virtually complete suppression at the 5-μg level. Deoxyribonuclease (DNAase) (100 μg/ml) and actinomycin D (15 μg/ml) caused little or no inhibition of ACTH or protein synthesis (data not given).

Discussion

The present study has broadly characterized the processes of radioactive proline incorporation into ACTH and mixed proteins, with a ribosomal system derived from anterior pituitary tissue. The labeled hormone was isolated by chemical methods used successfully with tissue slices (Adiga *et al.*, 1965), and was subsequently purified by paper electrophoresis. In the latter procedure, the radioactive polypeptide migrated identically with three ACTH reference standards. In addition, it may be mentioned that, as in earlier tissue slice experiments, labeled isoleucine was poorly utilized for ACTH biosynthesis, although it was well incorporated into protein. This amino acid is not a constituent of the bovine hormone molecule.

The incorporation of [^{3}H]proline into ACTH resembles the labeling process for protein from several standpoints: the requirements for ATP, Mg^{2+}, GTP, and an amino acid mixture; the pH optimum; the inhibitory effect of RNAase and puromycin; and particularly in the participation of a ribosomal and a pH 5 fraction. It seems probable that a messenger ribonucleic acid (m-RNA) is required, and contained in either (or both) of the subcellular components. Very likely aminoacyl-RNA synthetases, transfer ribonucleic acids (t-RNA's), and transferase enzymes are present in the pH 5 fraction. The possibility that polysomes are functional in ACTH formation is not excluded, and is receiving current attention.

It may be noted that cell-free systems were active in the syntheses of two other protein hormones; namely insulin (Wagle, 1965b) and thyroglobulin (Morais and Goldberg, 1966). These systems, like the present one, are ribosome dependent, as in the generally accepted pathway for protein synthesis. The chemical and biological identity of the biosynthetic ACTH is presently being confirmed by a number of procedures, and this information will be provided in a subsequent paper.

The high rate of labeled amino acid incorporation into proteins, observed in both adenohypophyseal slices and cell-free system, and sustained for relatively long periods of incubation, invite biosynthetic studies of the several protein hormones of this tissue. These problems are currently under investigation in our laboratory.

Acknowledgments

The generosity of Mr. Leonard F. Bennett, General Manager of the Hawaii Meat Co., in making available a routine supply of fresh beef pituitary glands is much appreciated. Thanks are also due to Dr. Carl Nash and Mr. George A. Halstead, Meat Inspection Division, U. S. Department of Agriculture for efficient cooperation.

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