Biosynthesis of Adrenocorticotropin and Protein in Slices of Bovine Anterior Pituitary Tissue*

P. Radhakantha Adiga, Isamu Uemura,† and Theodore Winnick

ABSTRACT: A small-scale procedure, developed for the isolation of adrenocorticotropin from anterior pituitary gland, has been employed in a study of the biosynthesis of the hormone in slices of this tissue. Upon incubation in Krebs-Ringer buffer, the slices were found to incorporate suitable ^{14}C-labeled amino acids efficiently into adrenocorticotropin and protein. The isolated radioactive hormone was identified by electrophoresis, chromatography, and bioassay. Analysis of chymotryptic digestion products revealed that isotope was distributed in various portions of the adrenocorticotropin molecule in accordance with the type of [^{14}C]amino acid originally employed. The biosynthesis of adrenocorticotropin was strongly suppressed by several inhibitors of protein formation: dinitrophenol, cyanide, azide, and puromycin. Chloramphenicol was without effect.

Considerable progress has been made during the past decade in elucidating the intricate mechanisms of protein biosynthesis. By contrast, relatively little is known of the biogenesis of natural polypeptides. A soluble enzyme catalyzes the polymerization of glutamic acid in *Bacillus lichenformis* (Leonard and Housewright, 1963). In *Staphylococcus aureus*, a series of enzymes promotes the stepwise formation of uridine nucleotide peptide (Ito and Strominger, 1960). Studies on the biosynthesis of several types of antibiotic polypeptides, such as the actinomycins (Katz and Weissbach, 1963) and bacitracins (Bernlohr and Novelli, 1963), have contributed toward the characterization of these processes in growing bacterial cultures. According to a recent report, a "light" (20,000 *g*) subcellular fraction of *B. lichenformis* promoted the addition of two terminal amino acids to a bacitracin precursor (Shimura *et al.*, 1964). In the case of the gramicidins and tyrocidines of *Bacillus brevis*, it was possible to demonstrate with cell-free preparations (Uemura *et al.*, 1963; Okuda *et al.*, 1964; Bodley *et al.*, 1964) that the biosynthesis of these peptides involved the participation of ribosomes, s-RNA, and soluble enzymes, as in protein formation.

The polypeptide hormones, ranging widely in molecular size, provide equally attractive model systems for the study of peptide bond synthesis in animal tissues. To date, the only *in vitro* study of this type appears to be that of Haller *et al.* (1964), dealing with the incorporation of [^{35}S]cysteine and [^{3}H]tyrosine into the vasopressin of the hypothalamohypophyseal complex of the guinea pig. Mention may also be made of tissue slice experiments on the biosynthesis of the low molecular weight protein insulin (Humbel, 1963; Mallory *et al.*, 1964).

The bovine adrenocorticotropin (ACTH)[1] molecule, with 39 amino acid residues (Li *et al.*, 1961), was selected for the present investigation.

```
 1  2  3  4  5  6  7  8  9  10 11
Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Try-Gly-Lys-
 12 13 14 15 16 17 18 19 20 21 22
Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-
 23 24 25 16 27 18 19 30 31 32
Tyr-Pro-Asp-Gly-Glu-Ala-Glu-Asp-Ser-Ala-
   33 34 35 36 37 38 39
GluNH2-Ala-Phe-Pro-Leu-Glu-Phe
```

The choice of ACTH was based upon: (a) the high metabolic activity of the hypophysis; (b) an adequate supply of freshly excised anterior lobe tissue; (c) the availability of purified ACTH preparations of known constitution; (d) satisfactory methods for the isolation of the hormone on a small scale; (e) good procedures for the separation and characterization of partial hydrolysis products of the polypeptide; (f) the interest associated with the comparative biochemistry of ACTH and with structure-function relationships; and (g) the unique role of ACTH in vertebrate physiology.

Some years ago, Melchior and Halikis (1952) showed that rat pituitary slices were highly active in incorporating [^{35}S]methionine into tissue proteins. More recently, Jacobowitz *et al.* (1963), in studying rats subjected to acute stress, found that ACTH was the most rapidly synthesized "protein" in the anterior lobe of the pituitary gland.

The main objectives of the present work were the determination of optimum conditions for amino acid incorporation into ACTH in slices of bovine anterior

* From the Department of Biochemistry and Biophysics, University of Hawaii, Honolulu. *Received September 8, 1964.* Supported by a research grant from the American Cancer Society (P-342). Contribution No. 43 of the Pacific Biomedical Research Center, University of Hawaii, Honolulu, Hawaii.

† On leave from the Osaka City University Medical School, Osaka, Japan.

[1] Abbreviation used in this work: ACTH, adrenocorticotropic hormone.

pituitary tissue, and the demonstration of labeling throughout the ACTH molecule. In addition, the effect of certain inhibitors of protein synthesis and of oxidative phosphorylation on ACTH biosynthesis was measured. It was pertinent, in most experiments, to follow the labeling of mixed tissue proteins, as well as of ACTH.

Experimental Procedures

Materials. A sample of purified ovine ACTH was kindly provided by Dr. C. H. Li, University of California. Porcine ACTH (120 international units/mg) was purchased from California Corp. for Biochemical Research. Crystalline γ-chymotrypsin was a product of Sigma Chemical Co. Radioactive amino acids, with the indicated specific activities in mc per mmole, were obtained from the following sources: DL-[1-^{14}C]-valine, 16.7, DL-[1-^{14}C]tryptophan, 6.6, and L-[U-^{14}C]-isoleucine, 8 from Calbiochem; L-[U-^{14}C]serine, 89, and L-[U-^{14}C]proline, 97 from Schwartz BioResearch, Inc.; DL-[1-^{14}C]leucine, 17 from New England Nuclear Corp.

Anterior Pituitary Tissue Slices. Glands were excised from cattle within a few minutes after slaughtering. The tissue was quickly dissected free of adhering structures, and washed in cold Krebs-Ringer bicarbonate buffer (*p*H 7.4) containing 2.16 g glucose per liter. After the gland was bisected longitudinally, the posterior lobe and adjacent region of each half were removed and discarded. The anterior tissue was then rapidly cut with a double-edged safety-razor blade (held by a hemostat) into slices about 0.5 mm in thickness. Some nine to eleven slices were conveniently obtained per gland. The pooled slices from several glands were washed in the above-mentioned buffer solution and used without delay in the incubation procedure. The foregoing process, carried out entirely in the cold, required only about 15–20 minutes from the death of the animals to the beginning of the incubation stage.

Incubation of Tissue Slices with [^{14}C]Amino Acids. Three to four slices of anterior pituitary tissue (totaling 80–100 mg, wet wt) were introduced into each of a series of 25-ml flasks, containing 4 ml of Krebs-Ringer buffer plus a specified radioactive amino acid. One μc of the L or 2 μc of the DL form of the latter was used, unless otherwise stated. The system was generally incubated for 8 hours (air atmosphere) at 37° in a rotary shaker. The incorporation process was terminated by adding 0.8 ml of 50% trichloroacetic acid to each flask.

Extraction of Labeled ACTH. The quantitative precipitation of ACTH (together with protein) by trichloroacetic acid and the selective solubility of the polypeptide in acid-acetone and in water (Dawson *et al.*, 1962; Li, 1956) form the basis of the following partial purification procedure: The denatured slices of each flask were transferred to a centrifuge tube and washed three times with 5-ml quantities of cold 5% trichloroacetic acid, recentrifuging each time. Each set of slices was then homogenized (glass-Teflon homogenizer) with 5 ml of trichloroacetic acid. After centrifugation, the previous washing procedure with trichloroacetic acid was performed three times on the acid-precipitated material in each tube. The precipitates were then washed with 5-ml portions of cold ether, and the washing was discarded. Each residue was extracted for 4 hours at room temperature with 4 ml of 65% aqueous acetone containing 10% glacial acetic acid (v/v), with intermittent stirring. Following centrifugation, the supernatant phase of each tube was separated and the sediment was reextracted briefly with 2 ml of the acid-acetone. The two extracts from each experiment were combined and evaporated to dryness at 35–40°. (The pellets were retained for isolation of protein.) The dry residues were individually suspended in 0.4-ml quantities of water, transferred to centrifuge tubes, and shaken briefly with 4 ml of chloroform (to solubilize lipids). Finally, the aqueous phase in each tube was separated by centrifugation.

Paper Electrophoresis of Labeled ACTH. Each of the above aqueous solutions (containing ACTH) was applied over a 5-cm width at the center of a strip of Whatman No. 1 paper, 40 cm in length. Conventional high-voltage electrophoresis, with cooling by 20° circulating water, was then employed. The buffer system was 1:10:89 (v/v) pyridine–glacial acetic acid–water. A current of 0.25 ma/cm was applied for 2 hours. The paper strip was then removed, air-dried, and stained with 0.5% aqueous bromophenol blue solution, containing 1% $HgCl_2$. Excess staining reagent was removed by thorough washing with 5% trichloroacetic acid. The paper was again air-dried. A discrete yellow band (A), about 2 cm wide, and displaced 8 cm from the origin toward the cathode, corresponded to the position of authentic ACTH (Figure 1). A narrow, faintly yellow band (B) could usually be detected at the origin.[2] The A band was cut from the paper, and extracted with three 1-ml quantities of 50% ethanol. The combined extracts were transferred to planchets for ^{14}C measurement.

Chymotryptic Digestion of Radioactive ACTH and Fractionation of Split Products. Twice the usual quantity of adenohypophyseal tissue slices (160 to 200 mg) was incubated for 8 hours with 20 μc of L-[^{14}C]amino acid (or 40 μc of racemic compound) in 8 ml of Krebs-Ringer buffer, in a 50-ml flask. The hormone was extracted and purified by the methods already described. The ACTH region of the paper electropherogram was eluted with 4–5 ml of water, and 1 mg of porcine ACTH carrier was added.[3] The solution was then lyophilized. The solid residue was dissolved in 0.5 ml of 0.2 M ammonium acetate–ammonia buffer (*p*H 9.0). γ-Chymotrypsin (10 μg) was added, and a 24-hour digestion at 37° was carried out. At the conclusion, the mixture was heated for 10 minutes at 100°.

[2] Upon exposure to ammonia vapors, this B band assumed a deep blue color.

[3] The peptide patterns of chymotryptic digestions were found to be essentially the same for bovine and for porcine ACTH.

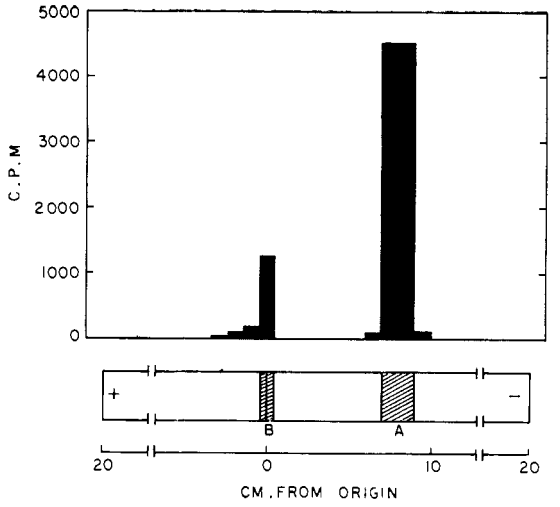

FIGURE 1: Electrophoresis of an extract of ACTH, labeled with [^{14}C]valine. The striped regions at the bottom represent staining along the paper strip.

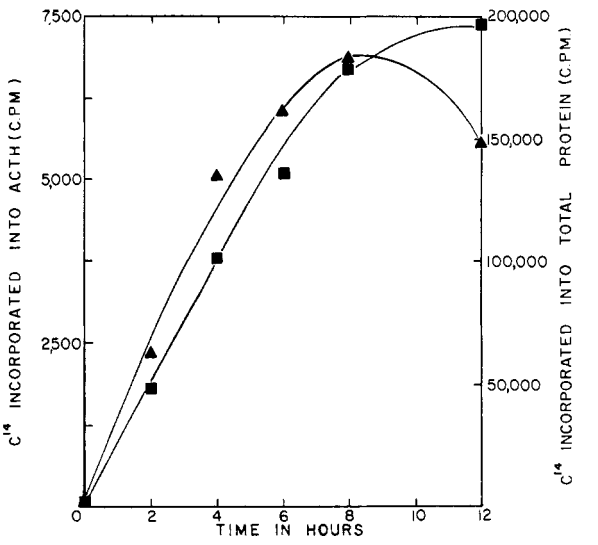

FIGURE 2: Rates of [^{14}C]valine incorporation into ACTH and into protein by anterior pituitary slices. ▲, ACTH; ■, protein.

Subsequently, paper electrophoresis was performed under the conditions described by Li *et al.* (1961). In this operation, a 12-cm-wide strip was employed, and subsequently a 1-cm band along either side of the completed electropherogram was cut out and developed with ninhydrin. With the help of these guides, the zones 1–7 (Figure 3) were easily identified. The guide strips were cut transversely at 1-cm intervals, and each segment was eluted into a planchet with 75% ethanol. In this way the ^{14}C content along the entire length of the electrophoretic strips could be determined. Radioactivity was found only in stained regions of these reference strips. The main (untreated) portions of each peptide fraction were eluted with 3 N NH_4OH and subjected to paper chromatography with appropriate solvent systems (Li *et al.*, 1961). The individual peptides resolved by these methods were detected by spraying with ninhydrin. Each stained peptide was eluted from the paper into a planchet, using 75% ethanol, preparatory to ^{14}C counting.

Isolation of Radioactive Protein. The above-mentioned pellet, derived from acid-acetone extraction of the trichloroacetic acid–precipitated tissue homogenate, was treated with hot and cold trichloroacetic acid, alcohol, and ether (Uemura *et al.*, 1963). Suitable portions of the resultant dry powder were dissolved in 2 N NH_4OH prior to plating on planchets.

Radioactivity Measurements. Samples were dried as uniform thin layers on metal planchets, and counted in the gas-flow Geiger counter. Self-absorption corrections were applied when necessary. Zero-time corrections were quite small, of the order of 0.01% of active incorporation values.

Bioassay of ACTH. The *in vitro* method described by Saffran and Schally (1955) and by Rerup (1958), based on the stimulation by ACTH of steroid production in rat adrenal slices, was employed.

Results

Purity of Isolated Labeled ACTH and Establishment of Identity. Figure 1 shows a typical paper electrophoretic pattern of a radioactive ACTH preparation derived from bovine anterior pituitary slices after incubation in the presence of an isotopic amino acid. It may be seen that the bulk of the ^{14}C (approximately 75%) was recovered in a narrow band (A) about 8 cm from the origin. There was a smaller proportion of the radioactivity in the B region. No ^{14}C could be detected elsewhere along the paper strip. With samples of authentic ovine or porcine ACTH, staining was observed in the identical A position, but not in the B zone. Qualitatively similar electropherograms were obtained when labeled leucine, proline, serine, or tryptophan were used in place of valine in the incubation procedure. The small component consistently found at the origin was probably protein. Its ^{14}C content fluctuated in accordance with the labeling observed in tissue protein under various conditions.

The radioactive ACTH appeared to be homogeneous by further tests: A mixture of the eluted A material and purified (ovine) hormone was not resolved upon reelectrophoresis. Likewise, in descending paper chromatography, the same mixture yielded a single spot with either of two solvent systems: 2:2:1 butanol–acetic acid–water (R_F 0.72) and 30:60:20:24 1-butanol–acetic acid–pyridine–water (R_F 0.91).

Final confirmation of the hormonal nature of the A band was obtained by bioassay. In several experiments, the eluates of this material had potencies corresponding to 1–2 international units/100 mg of anterior pituitary tissue.

Time Course and Optimum Conditions for Labeling of ACTH and Protein. Figure 2 shows that the incorporation of isotopic valine into ACTH of anterior

pituitary tissue continued actively for about 8 hours. Thereafter the recovery of ^{14}C in the polypeptide declined. The reason for this terminal fall (observed in repeated experiments) is not clear. The labeling of protein increased up to at least 12 hours. The quantity of ^{14}C incorporated into tissue protein was about 25 times greater than that for polypeptide. This ratio varied considerably in different preparations.

No further improvement in biosynthesis was achieved by varying the *p*H of the buffer solution, substituting phosphate for the bicarbonate, replacing the air atmosphere with oxygen, or altering the quantity of tissue employed. The omission of glucose in the external medium resulted in a 50% decrease in labeling of the hormone and protein.

Comparison of Various [^{14}C]*Amino Acids.* Several amino acids, differing widely in their relative abundance in the ACTH molecule, were tested in the tissue-slice system (Table I). These comparisons were complicated by such factors as cell permeability, sizes of endogenous amino acid pools, and possible metabolic transformations. However, a fairly good correlation was observed between the efficiency of utilization of amino acids and their abundance in ACTH. Thus proline, which occurs to the extent of 4 residues in the polypeptide structure (Li *et al.*, 1961), was utilized to the greatest degree for hormone biosynthesis, followed by valine and serine (3 residues each). Leucine and tryptophan (1 residue each) were incorporated to a considerably lesser degree, while isoleucine, which is not a constituent of the hormone molecule, was not incorporated to a measurable extent.

In the case of tissue protein, valine and leucine were the best utilized amino acids, followed by isoleucine, proline, serine, and tryptophan. The ratio of total radioactivity appearing in proteins, as compared to ACTH, varied widely: from approximately 15 with [^{14}C]proline to more than 200 with [^{14}C]leucine.

TABLE I: Utilization of Different Isotopic Amino Acids for ACTH and Protein Biosynthesis.

[^{14}C]Amino Acid Used	Per Cent of Total ^{14}C Recovered[a] In ACTH	In Protein
L-Proline	0.51	8.1
DL-Valine	0.33	32.3
L-Serine	0.30	5.9
DL-Leucine	0.07	16.0
DL-Tryptophan	0.06	3.9
L-Isoleucine	0.00	8.5

[a] Based on the quantity of radioactive L isomer employed.

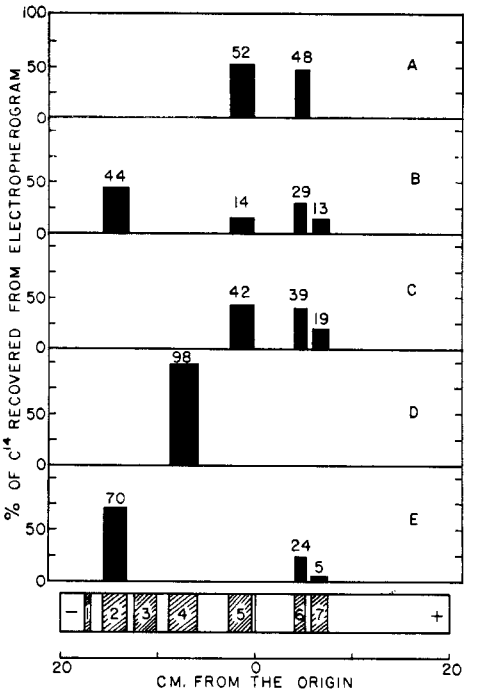

FIGURE 3: Partial resolution by paper electrophoresis of radioactive peptide fragments of ACTH, following digestion with chymotrypsin. The [^{14}C]amino acids employed in separate experiments were: (A) DL-leucine, (B) L-proline, (C) L-serine, (D) DL-tryptophan, (E) D valine.

Analysis of Partial Hydrolytic Products of ACTH. In order to provide evidence tending to indicate *de novo* biosynthesis, it was pertinent to determine whether ^{14}C was distributed in various regions of the ACTH molecule after incubation of anterior pituitary slices with several isotopic amino acids. The first stage in this process was the digestion of labeled ACTH preparations with chymotrypsin and the electrophoretic separation of the hydrolytic pr cts into several groups of peptides. The fractionation pattern obtained (lower portion of Figure 3) was identical with that described by Li *et al.* (1961). Radioactivity measurements were performed on peptide fraction numbers 1–7 in five separate experiments, each employing a different [^{14}C]amino acid. It may be seen (Figure 3) that the total recovery of isotope was virtually quantitative in each case. With radioactive leucine as the labeling agent, the ^{14}C of the chymotryptic digest of ACTH was about equally distributed between fractions 5 and 6. With [^{14}C]proline, radioactivity was found in fractions 2, 5, 6 and 7. With [^{14}C]serine and valine, the labeling was localized in fractions 5, 6, 7, and 2, 6, 7, respectively. In the [^{14}C]tryptophan experiment, all of the isotope was concentrated in fraction 4.

The peptide fractions described in Figure 3 were completely resolved by paper chromatography (Li *et al.*, 1961), and all of the resulting individual components were analyzed for ^{14}C. Only those fragments

TABLE II: Distribution of Radioactivity in Individual Peptides following Electrophoretic and Chromatographic Resolution of Chymotryptic Digest of Labeled ACTH.

[^{14}C]Amino Acid Employed	Electrophoretic Zone Number	Peptides Isolated by Paper Chromatography				Per Cent of Radioactivity Recovered	
			R_F				
		Designation[a]	Solvent[b] I	Solvent[c] II	Amino Acid Sequence[a]	From Electrophoretic Zone	From Original Chymotryptic Digest of ACTH
Tryptophan	4	c	0.54		8 9 Arg-Try	97	96
Leucine	5	e	0.87		34 37 Ala...Leu	97	51
	6	b		0.31	21 37 Lys...Leu	96	48
Serine	5	b	0.25		3 7 Ser...Phe	41	17
		c	0.55		1 2 Ser-Tyr	51	21
		d	0.70		Not known	8	4
	6	a		0.19	21 31 35 Lys...Ser...Phe	52	21
		b		0.31	21 31 37 Lys...Ser...Leu	47	18
	7	a		0.05	21 31 33 Lys...Ser...GluNH$_2$	97	19
Valine	2	a	0.07		10 13 15 Gly...Val...Lys	34	24
		b	0.13		16 20 Lys...Val	22	16
		c	0.28		17 20 22 Arg...Val...Val	44	31
	6	a		0.19	21 22 35 Lys-Val...Phe	52	13
		b		0.31	21 22 37 Lys-Val...Leu	47	11
	7	a		0.05	21 22 33 Lys-Val...GluNH$_2$	96	5
Proline	2	a	0.07		10 12 15 Gly...Pro...Lys	35	16
		b	0.13		16 19 20 Lys...Pro-Val	31	14
		c	0.28		17 19 22 Arg...Pro...Val	33	15
	5	e	0.87		34 36 37 Ala...Pro-Leu	98	13
	6	a		0.19	21 24 35 Lys...Pro...Phe	39	11
		b		0.31	21 24 36 37 Lys...Pro...Pro-Leu	60	17
	7	a		0.05	21 24 33 Lys...Pro...GluNH$_2$	98	13

[a] Li *et al.* (1961). [b] Solvent I, 30:20:6:24 1-butanol–pyridine–acetic acid–water (24 hours). [c] Solvent II, 4:1:5 1-butanol–acetic acid–water (16 hours).

containing measurable amounts of isotope have been included in Table II. These total 21 in number, ranging in size from dipeptides to an oligopeptide with 16 amino acid residues. The electrophoretic values (the next-to-last column) provide more detailed evidence that the *in vitro* incorporation process led to labeling throughout the ACTH molecule.

It was of particular interest to compare the observed distribution of a given isotopic amino acid in different regions of the hormone molecule with theoretical expectations, assuming a uniform labeling process in biosynthesis. The data in the last column of Table II were used for this purpose. The radioactive tryptophan and leucine experiments need not be considered here, since only one moiety of each amino acid was present in the hormone. In the case of serine, residues of this amino acid occur in positions 1, 3, and 31 along the polypeptide chain. The corresponding quantities of ^{14}C found in these sites are given by the radioactivity in peptides 5c (21%), 5b (17%), and 6a + 6b + 7a (58%). Uniform distribution of ^{14}C would have required $33^1/_3\%$ of the total isotope in each serine sequence. It may be noted that a minor portion of the isotope was present in a peptide (5d) of unknown constitution.

In the isotopic valine and proline experiments, the interpretations were complicated by labeling in *two* positions of one of the peptides isolated, the 2c and 6b fragments, respectively. The most valid procedure was to contrast the total radioactivity in this doubly labeled region with that in other parts of the ACTH molecule. Thus, with [^{14}C]valine, 24% of the isotope was found in position 13 (peptide 2a), and 76% in positions 20 plus 22 (the remaining five peptides). The corresponding percentages anticipated according to uniform labeling would be $33\ ^1/_3$ and $66^2/_3$. Similarly with [^{14}C]proline, the per cent distribution of isotope in positions 12 (2a), 19 (2b + 2c), and 24 plus 36 (5e + 6a + 6b + 7a) was 16, 29, and 54, respectively. The uniform labeling mechanism would have given 25, 25, and 50% for the three sites of [^{14}C]proline incorporation.

Influence of Inhibitors of Protein Synthesis on [^{14}C]*-Amino Acid Incorporation into ACTH.* In seeking exploratory information on the mechanism of ACTH biosynthesis, it was of interest to test the effects of well-known inhibitors of respiration and oxidative phosphorylation in the pituitary-slice system. The results in Table III show that dinitrophenol, azide, and cyanide strongly suppressed polypeptide as well as protein formation. In fact, ACTH synthesis was the more sensitive process at high concentrations of the three inhibitors.

In view of the wide current usage of antibiotic substances in studies on the mechanism of protein biosynthesis, two of these agents, chloramphenicol and puromycin, were tried at varying concentrations with anterior pituitary tissue. The results in Figure 4 show that a concentration of 0.5 μmole of puromycin per ml of buffer solution gave approximately 95 and 80% inhibition of ACTH and protein biosynthesis, respectively. Chloramphenicol, a strong antagonist of protein synthesis in bacterial cells, was found to be completely without effect on either ACTH or protein formation in anterior pituitary slices, even at concentrations up to 100 μg/ml of buffer. This observation agrees with findings for mammalian cell-free systems (Von Ehrenstein and Lipmann, 1961; Rendi and Ochoa, 1962).

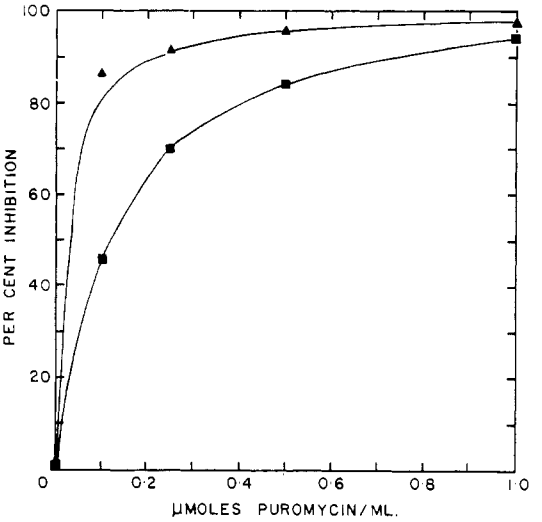

FIGURE 4: The effect of puromycin on the incorporation of [^{14}C]valine into ACTH and protein in anterior pituitary slices. ▲, ACTH; ■, protein.

TABLE III: Effect of Dinitrophenol, Azide, and Cyanide on [^{14}C]Valine Incorporation into ACTH and Protein of Anterior Pituitary Slices.[a]

Inhibitor Added to Incubation Medium (μmoles/ml)		Radioactivity	
		ACTH	Protein
Dinitrophenol:	0.1	51	44
	1	13	11
	10	0.5	5
Sodium azide:	10	53	37
	100	0.5	2
KCN	1	52	59
	10	6	22
	100	1	10

[a] The values are expressed relative to those of control experiments (without inhibitors), taken as 100.

Discussion

The present experiments confirm the findings of other investigators (Melchior and Halikis, 1952; Jacobowitz *et al.*, 1963) that anterior pituitary tissue is unusually active in ACTH and protein biosynthesis.

The slices retained their potency for a remarkably long period of time. Assuming a concentration of 60 mg of ACTH (Li, 1956) and approximately 16 g of protein per 100 g of fresh anterior lobe tissue (Melchior and Halikis, 1952), the mixed proteins were about 260 times as abundant as the polypeptide hormone. However, the observed ratios of total ^{14}C incorporation into protein, as compared to ACTH, were much less than 260 with most of the isotopic amino acids tested (Table I). This conclusion agrees with the observation (Jacobowitz *et al.*, 1963) that ACTH was synthesized more rapidly than were various cellular proteins of the adenohypophysis.

The techniques used to isolate and characterize the labeled ACTH leave little doubt as to its biochemical identity or its purity. It is significant, in this connection, that L-[^{14}C]isoleucine (which is not a constituent of the hormone molecule) was not incorporated into the ACTH isolated after incubation. The recovery of biological potency was low, as compared to reported values (Li, 1956). This may reflect the sensitivity of the hormonal activity to oxidation (Li, 1956). Such losses may well have occurred during the isolation and purification methods employed in the present work. No attempt was made to determine whether the bovine hormone consisted of mixed molecular species, as in the case of ovine ACTH (Pickering *et al.*, 1963). The analysis of the chymotryptic digestion products has clearly shown that the incorporated [^{14}C]amino acids were distributed throughout the polypeptide chain, with isotope appearing in all expected positions. No other radioactive fractions were detected in these experiments, thereby further substantiating the purity of the isolated labeled ACTH. These data provide evidence consistent with *de novo* synthesis of the hormone (Campbell, 1961). The available information (Chantrenne, 1961) seems still inadequate to permit a decision with regard to uniform versus nonuniform labeling in protein biosynthesis. The analysis of ^{14}C-distribution patterns in the case of ACTH suggests irregularities, particularly in the isotopic serine experiment of Table II. However, the data are not considered sufficiently extensive to permit a clear choice between the two views.

The inhibition of ACTH formation by dinitrophenol, cyanide, and azide resembles the results in early studies of protein biosynthesis in liver and intestinal slices (Chantrenne, 1961) and reflects the need for an energy source (presumably ATP). As in the case of the gramicidin and tyrocidine polypeptides of *B. brevis* (Okuda *et al.*, 1964), the synthesis of ACTH was strongly inhibited by puromycin, a classic antagonist of protein biosynthesis. This observation is not surprising in view of the finding that vasopressin formation was also depressed by this same antibiotic (Haller *et al.*, 1964). It may be pointed out at this juncture that ACTH was found distributed between microsomal and supernatant fractions in porcine anterior pituitary preparations (Brown and Ulvedal, 1960). The possibility therefore exists that the Zamecnik cycle of reactions governs the biosynthetic pathway of the several pituitary peptide hormones. Studies with cell-free preparations of hypophyseal tissue may be expected to elucidate this intriguing question.

Acknowledgments

We wish to thank Dr. C. N. H. Long of Yale University for valuable discussions on certain aspects of the research. The efficient cooperation of Dr. Philip G. Papish and Mr. George A. Halstead, Meat Inspection Division of the U.S. Department of Agriculture; and Mr. Herbert E. Welhener, General Manager of the Hawaii Meat Co., in making available a supply of fresh pituitary glands is gratefully acknowledged. A sample of highly purified ACTH was kindly provided by Dr. C. H. Li, University of California.

References

Bernlohr, R. W., and Novelli, G. D. (1963), *Arch. Biochem. Biophys. 103*, 94.

Bodley, J. W., Uemura, I., Adiga, P. R., Okuda, K., and Winnick, T. (1964), *Biochemistry 3*, 1492.

Brown, J. H. V., and Ulvedal, F. (1960), *Endocrinology 66*, 175.

Campbell, P. N. (1961), *Protein Biosyn. Symp. Wassernaar, Neth., 1960*, 19.

Chantrenne, H. (1961), *in* Biosynthesis of Proteins, New York, Pergamon, chap. 4.

Dawson, R. M. C., Elliott, D. C., Elliott, W. H., and Jones, K. M. (1962), *in* Data for Biochemical Research, London, Oxford, p. 164.

Haller, E., Takabatake, Y., and Sachs, H. (1964), *Federation Proc. 23*, 205.

Humbel, R. E. (1963), *Biochim. Biophys. Acta 74*, 96.

Ito, E., and Strominger, J. L. (1960), *J. Biol. Chem. 235*, PC 5.

Jacobowitz, D., Marks, B. H., and Vernikos-Denellis, J. (1963), *Endocrinology 72*, 592.

Katz, E., and Weissbach, H. (1963), *J. Biol. Chem. 238*, 666.

Leonard, C. G., and Housewright, R. D. (1963), *Biochim. Biophys. Acta 73*, 531.

Li, C. H. (1956), *Advan. Protein Chem. 11*, 101.

Li, C. H., Dixon, J. S., and Chung, D. (1961), *Biochim. Biophys. Acta 46*, 324.

Mallory, A., Smith, G. H., and Taylor, K. W. (1964), *Biochem. J. 91*, 484.

Melchior, J. B., and Halikis, M. N. (1952), *J. Biol. Chem. 199*, 773.

Okuda, K., Uemura, I., Bodley, J. W., and Winnick, T. (1964), *Biochemistry 3*, 100, 108.

Pickering, B. T., Andersen, R. N., Lohmar, P., Birk, Y., and Li, C. H. (1963), *Biochim. Biophys. Acta 74*, 763.

Rendi, R., and Ochoa, S. (1962), *J. Biol. Chem. 237*, 3711.

Rerup, C. (1958), *Acta Endocrinol. 29*, 83.

Saffran, M., and Schally, A. V. (1955), *Endocrinology 56*, 523.

Shimura, K., Sasaki, T., and Sugawara, K. (1964), *Biochim. Biophys. Acta 86*, 46.
Uemura, I., Okuda, K., and Winnick, T. (1963), *Biochemistry 2*, 719.
Von Ehrenstein, G., and Lipmann, F. (1961), *Proc. Natl. Acad. Sci. U.S. 47*, 941.

Amino Acid Incorporation into Protein by Cell-free Preparations from Rat Skeletal Muscle. III. Comparisons of Activity of Muscle and Liver Ribosomes

James R. Florini and Charles B. Breuer

ABSTRACT: Comparisons of amino acid incorporation into protein by liver versus muscle ribosomes have been made under conditions in which ribosomes were limiting. Liver ribosome were two to three times as active as muscle ribosomes; addition of synthetic messenger ribonucleic acid (polyuridylic acid) abolished this difference. Similar observations were made when either liver or muscle was the source of soluble RNA, transfer enzymes, and *p*H 5 precipitate, and when either free amino acid or amino-acyl-s-RNA was the labeled precursor. There was no evidence that endogenous inhibitors lowered the activity of the muscle preparation. On the basis of these and earlier results, it is concluded that the large differences in protein labeling by liver and muscle *in vivo* and *in vitro* are attributable both to the lower ribosome content of muscle and to the lower activity of the isolated muscle ribosomes which is probably a result of their lower content of m-RNA.

Our primary interest in studying amino acid incorporation into protein by rat skeletal muscle preparations *in vitro* has been the elucidation of mechanisms controlling protein synthesis in this tissue. The previous papers in this series (Florini, 1962; Florini, 1964; Breuer *et al.*, 1964) presented characterizations of the muscle system at various levels of purification. In general, the muscle system was similar to but far less active than analogous preparations from liver, even when activity was expressed on the basis of the RNA content of the microsome fractions. We have suggested (Florini, 1964) that muscle microsomes were less active because they contained either less messenger RNA or more contaminating nonribosomal RNA. This paper presents evidence which supports the former suggestion, although the possibility remains that entrapped cytoplasmic RNA was partially responsible for the lower activity of the muscle microsomes when incorporation was expressed in terms of RNA content of the microsomes.

Experimental

Materials and Methods. The materials, techniques, and procedures used in this study were described in detail in paper II in this series (Breuer *et al.*, 1964). Ribosomes of high purity (260/280 mμ absorbancy ratios of 1.75 to 1.83) were prepared from muscle or liver of 18-hour starved normal male rats (Carworth Farms CFN strain) by treating the resuspended microsomal pellet with 0.5% Lubrol WX and 1.0% deoxycholate followed by centrifugation through a layer of 1.0 M sucrose. The 20–35% and 50–65% saturated $(NH_4)_2SO_4$ fractions of the *p*H 5-soluble portion of the 105,000 $\times$ *g* supernatant were combined, passed through Sephadex G-25, and used as the transfer enzyme preparation. s-RNA was charged with the specified labeled amino acid by the method of Bloemendal *et al.* (1962). Contents of incubation tubes are specified in the legends to the tables. After incubation for 15 minutes at 37°, samples were precipitated with 5% trichloroacetic acid, washed by the Siekevitz (1952) procedure, and hydrolyzed, and the specific radioactivity of the resulting amino acid solution was determined. Results are expressed as $\mu\mu$moles of amino acid incorporated or transferred into protein, although it is apparent that appreciable dilution of the very highly labeled amino acids occurred when the tracer was added to the relatively large quantities of amino-acyl-s-RNA present in the incubation mixture (Table I) or used for the charging of unstripped s-RNA. Thus the observed labeling represents the lower limit of amino acid incorporation actually occurring; it is probably several orders of magnitude lower than the

* From the Biochemistry Research Department, Biochemical Research Section, Lederle Laboratories, Pearl River, N. Y.
Received October 5, 1964.