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Adrenocorticotrophin and Adrenal Protein Synthesis[1]

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ABSTRACT. *In vitro* protein synthesis by a cell-free preparation (15,000 ×g supernatant fraction) of pig adrenals was studied, measuring glycine-^{14}C and *l*-leucine-^{14}C incorporation into protein. Adrenocorticotrophin administered to rats *in vivo* caused a significant increase in the total activity of nondialyzable "supernatant factor" stimulating amino acid incorporation into protein. The specificity of the observed effect for adrenocorticotrophin was confirmed by experiments with both a synthetic tricosapeptide and a highly purified preparation obtained from natural sources. The "supernatant factor" was nondialyzable, had an approximate molecular weight of 10–14 ×10^4, and was inactivated by incubation at 55 C and also by incubation with trypsin. It was not inactivated by ribonuclease. Nucleic acids prepared by several methods were inactive. The "supernatant factor" could be purified by diethylaminoethyl cellulose chromatography, and chemical analysis revealed the protein nature of the material with virtually no ribonucleic acid present in the preparation. It is tentatively concluded that this material is one of the transfer enzymes involved in protein synthesis, several other possibilities having been excluded. (*Endocrinology* **76**: 745, 1965)

THE ADMINISTRATION of adrenocorticotrophin *in vivo* (1) increased the activity of a nondialyzable factor in the 105,000 ×*g* supernatant of rat adrenals that stimulated the incorporation of glycine-^{14}C into protein when added to a cell-free preparation from the adrenals of control rats. Evidence has been presented (1) indicating that this stimulation occurs at a step after the formation of amino acyl-sRNA. Since adrenal growth is such an integral part of pituitary-adrenal interrelationships, it was felt of interest to characterize further this "supernatant factor."

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Materials

The preparations of adrenocorticotrophin were generously donated as follows: Corticotrophin-gel, porcine (80 USP units/ml) lots SB 543, SB 849, PR 583, by Dr. F. G. McMahon, The Upjohn Co.; Oxycel adrenocorticotrophin, bovine (86 USP units/mg) batch CN 145 by Dr. E. A. Laso-Wasem, Wilson Co.; and N-terminal tricosapeptide synthetic (109 USP units/mg) by Dr. K. Hofmann, University of Pittsburgh.

Glycine-U-^{14}C and *l*-leucine-U-^{14}C were purchased from Nuclear-Chicago, and the specific activity of each was diluted to 2.79 μc/μmole by the addition of carrier. Other materials were obtained as follows: phosphoenolpyruvate (purchased as a barium salt and converted to the potassium salt), disodium-adenosine triphosphate, adenosine-3′,5′-cyclic phosphoric acid, trypsin, and diethylaminoethyl cellulose (capacity: 0.9 mEq/g; mesh: medium, 19t D 62-13-240) from Sigma Chemical Co.; trisodium guanosine triphosphate, crystalline lactic dehydrogenase (2 mg/ml) and pyruvate kinase (10 mg/ml) from Calbiochem.; ribonuclease from Worthington Biochemical Co.; dialysis tubing (No. 4465-A2) from A. H. Thomas Co.; pig adrenals from Northeast Packing Co.,

TABLE 1. Effect of addition of different substances to the assay system on the glycine-^{14}C incorporation

Addition*	% Change
Amino acid mixture	0
Adenosine-3′,5′-cyclic phosphoric acid 10^{-4}M	0
Sodium ascorbate 1.6×10^{-2}M	0
57 μg PEP kinase	0
114 μg PEP kinase, 20 μmoles PEP and 2 μmoles ATP	0
.25 μmole GTP	0
7×10^{-5}M Glutathione	−12
7×10^{-4}M Glutathione	−33
7×10^{-3}M Glutathione	−66
0.4 ml 15,000 × *g* supernatant of pig adrenals	+100

* All additions were added in 0.4 ml of Tris-HCl buffer containing sucrose and ions at the concentrations noted in the text. The pH of the solution was checked and adjusted to 7.5 when necessary.

Somerville, Mass.; male Sprague-Dawley rats (250–300 g) from Charles River Breeding Co.

Methods

The 15,000×*g* supernatants obtained from pig adrenals were employed as the basic system for testing the stimulation of amino acid incorporation into proteins. The adrenals were placed in ice-cold 0.9% NaCl approximately 20 min after death, dissected free of adherent fat tissue, and frozen (−10 C). Prior to each incubation, pig adrenals were homogenized in medium A (200 mg/ml) of Littlefield and Keller (2) for 20 sec in a Virtis blender and then for 3–5 min in a glass homogenizer equipped with a motor-driven Teflon pestle. The supernatant fluids obtained after 15 minutes' centrifugation of the homogenates at 15,000×*g* and 5 C contained microsomes and the soluble fraction (3, 4). Amino acid incorporation into protein did not change after storage of the adrenals at −10 C for more than 2 months.

Rat adrenals were removed immediately after death, adherent fat tissue was removed, and the adrenals were homogenized in medium A in a glass homogenizer. The 120 min×105,000×*g* supernatant was obtained as previously described (1).

The complete incubation mixtures contained in a total volume of 1.25 ml the following: 1 μc (0.36 μmole) glycine-^{14}C, 10 μmoles phosphoenolpyruvate, 1 μmole adenosine triphosphate, 0.25 μmole guanosine triphosphate, 57 μg dialyzed pyruvate kinase, 0.4 ml of the 15,000×*g* supernatant of pig adrenals, 0.005M Mg^{++}, 0.017M K^{+}, 0.039M Tris-HCl buffer, pH 7.5, 0.17M sucrose, and 0.4 ml of the test solution to be assayed. These mixtures were incubated at 37 C for 60 min (unless otherwise stated). The protein concentration was measured (5), and the radioactivity of the residue insoluble in hot trichloroacetic acid was determined by means of a liquid scintillation counter (Nuclear-Chicago) as previously described (1). Differences between groups of values were analyzed for significance by the *t* test (6).

Sucrose density gradients containing 0.025M KCl, 0.005M $MgCl_2$, 0.05M Tris-HCl buffer, pH 7.5, were prepared by a modification[3] of the method of Britten and Roberts (7). The gradients were centrifuged for 1–2 hr at 37,500 rpm using the swinging bucket rotor SW-39 and the Spinco Model L ultracentrifuge. The linearity of gradients prepared in this manner was checked by the addition of a dye to the sucrose solution with the higher concentration. Two hundred to 400 μl of test solutions was layered very carefully on top of such gradients. After centrifugation, the tubes were punctured at the bottom and fractions were collected dropwise in the conventional manner.

Results

Omission of adenosine triphosphate and the regenerating system resulted in only 11% (22 cpm) of the glycine-^{14}C incorporation obtained in the complete system (192 cpm). Dialysis of the pig adrenal 15,000×*g* supernatant for 18 hours *vs.* medium A resulted in the loss of ability to incorporate amino acids into protein, but this ability was restored by the addition of 1 μmole of each of 19 amino acids[4] to the incubation mixture.

[3] We wish to thank Mr. S. Slapikoff for introducing us to the unpublished modification of Slapikoff, S., J. M. Fessenden, and K. Moldave, of the method for the preparation of sucrose gradients of Britten and Roberts (12).

[4] Ala, try, lys, val, his, ser, thr, arg, ileu, phe, glu-NH_2, leu, asp, cySH, cyS, glu, met, pro, tyr were dissolved in 0.01N HCl (10 μmoles/ml) and neutralized immediately before the incubations. Under these conditions all amino acids, except tyr and cyS, were completely soluble.

The effects of various additions to the complete assay system are presented in Table 1.

The addition of the 105,000 $\times g$ supernatant from adrenals of control rats increased the incorporation of glycine into protein (Table 2) compared with the addition of an equal volume of buffer ($p < 0.001$). The addition of the 105,000 $\times g$ supernatant from an equal weight of adrenals from rats treated with adrenocorticotrophin (7 $\times$ 20 USP units adrenocorticotrophin-gel, Upjohn, intramuscularly over 3 days) results in a significantly greater increase in incorporation ($p < 0.01$).

Dog adrenal 15,000 $\times g$ supernatant was equally responsive to the addition of rat adrenal 105,000 $\times g$ supernatant, whereas a 15,000 $\times g$ supernatant from rat liver did not show increased incorporation when rat adrenal 105,000 $\times g$ supernatant was added.

The specificity of the observed effect as a response to adrenocorticotrophin and not to a possible contamination in the commercial preparation was checked by studies with the synthetic N-terminal tricosapeptide of Hofmann (8). The adrenal content of the "supernatant factor" was increased significantly (Table 3) by the infusion of the tricosapeptide. Under the same conditions, the infusion of 1.5 units of Oxycel-adrenocorticotrophin increased the adrenal content of the "supernatant factor." The weights of the adrenals of rats infused with adrenocorticotrophin showed a slight increase (5–10%) *vs.* control rats, but it was not significant.

TABLE 2. Effect of 105,000 $\times$ g supernatant of rat adrenals on glycine-U-^{14}C incorporation into protein

Source of test solution	cpm in protein ± SE
Buffer	202 ± 10
Control adrenals	618 ± 30.8
ACTH-treated rats	916 ± 40.2

TABLE 3. Effect of infusion of synthetic tricosapeptide on supernatant factor in adrenals of hypophysectomized rats

Test solution	cpm in protein ± SE
0.4 ml of medium A	183 ± 3
0.35 ml of 105,000 $\times$ g supernatant of control rats	356 ± 10.7
0.35 ml of 105,000 $\times$ g supernatant of treated rats	527 ± 19.5

Hypophysectomized animals were provided with 0.9% NaCl, water and Purina laboratory chow *ad lib.* for 10 days. Five days prior to the infusion, im treatment with 0.1 mg/day desoxycorticosterone acetate (4-pregnene-21-ol-3,20-dione) for suppression of the zona glomerulosa of the adrenal (26, 27) and with 0.01 mg/day Decadron phosphate (16α-methyl-9α-fluoro-1,4-pregnenedione-11β,17α,21-triol-3,20-dione) for maintenance (28) was begun; the latter dose was doubled on the morning of the operation. By that time the rats had lost 5–10% of their weight and had polydipsia. The weights of the adrenals were half of that found in normal adrenals. After catheterization of the right jugular vein with a polyethylene catheter (PE 20–50) under ether anesthesia, the rats were kept in restraining cages. By means of a continuous infusion pump, 6.5 ml 0.9% NaCl containing 18 μg of the tricosapeptide (equivalent to approximately 2 USP units adrenocorticotrophin) was infused into each of 4 rats over a 315-min period. Four control hypophysectomized rats were identically treated except that the infused solution did not contain the tricosapeptide. After the infusion period, the rats were decapitated and the adrenals were prepared and collected in cold 0.9% NaCl. Four pairs of control adrenals weighed 39 mg and were homogenized with 0.78 ml of medium A, and 4 pairs of adrenals from the tricosapeptide-infused rats weighing 41 mg were homogenized with 0.82 ml of medium A, both for 90 sec by means of a glass homogenizer. The supernatant fluid after centrifugation for 120 min at 105,000 $\times$ g (+4 C) was kept on ice overnight.

The "supernatant factor" sediments in sucrose gradients upon centrifugation in front of that area containing the material which gave the highest absorption at 260 and 280 mμ as shown in Fig. 1. The smaller amount of "supernatant factor" found in the 105,000 $\times g$ supernatant of an equal weight of control rat adrenals (*cf* Table 2) sediments identically in sucrose gradients.

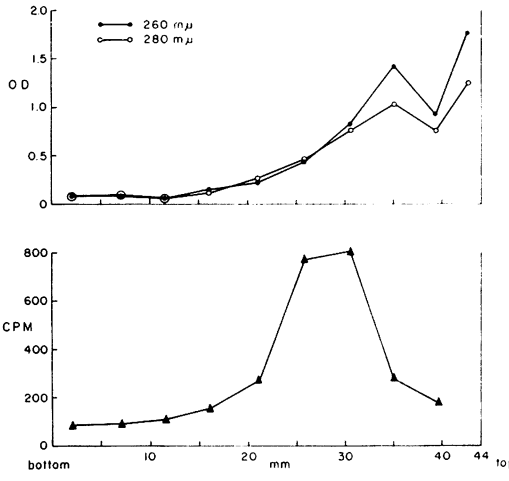

FIG. 1. Distribution of activity of 105,000 × *g* supernatant of adrenals from rats treated with adrenocorticotrophin after centrifugation on a sucrose gradient. The supernatant from 200 mg of adrenal was layered on a 6–20% sucrose gradient and centrifuged for 19 hr at 37,500 rpm, t = +1 C. Triangles represent glycine-^{14}C incorporation into protein upon addition of 0.3 ml of each fraction of the density gradients as test solution to the assay system. In the upper half of the figure, the optical density of 0.05 ml of each density gradient fraction diluted 1:8 is shown. In the lower half of the figure, no point is present for the upper area of the gradient. This fraction in several experiments decreased incorporation to variable levels below that obtained with the basal test solution.

Martin and Ames (9) have suggested the use of purified proteins of known molecular weights as references for the determination of an approximate molecular weight of an unknown substance. The 105,000 × *g* supernatant of adrenals from rats treated with adrenocorticotrophin was centrifuged on sucrose gradients 5–20% and 5–13%. Pyruvate kinase and lactic dehydrogenase were centrifuged simultaneously on separate gradients. The latter gradient was employed to improve the separation of the stimulating factor, but was without significant effect. Centrifugation was performed for 16.5 hours at 37,500 rpm and +1 C. Glycine-^{14}C incorporation was measured upon addition of 0.4 ml of each fraction of one density gradient as test solution to the assay system. The protein content of consecutive 0.1 ml fractions of the other gradients containing pyruvate kinase or lactic dehydrogenase was determined. Calculations were carried out as described by Martin and Ames using data from the literature (10–12) and the data obtained in the present experiments. The results indicate a molecular weight of 1.0–1.4 × 10^5 and an $S_{20}W$ of 6 to 7 for the stimulating factor.

The "supernatant factor" of adrenals from control or rats treated with adrenocorticotrophin was stable upon preincubation for 2 hours at 37 C and for 15 minutes at 45 C prior to addition to the assay system. The activity from both sources was completely destroyed upon preincubation for 15 minutes at 55 C or higher temperature.

The effect of trypsin on the "supernatant factor" was studied. Trypsin was shown to inhibit glycine incorporation into protein by the assay system, and it was necessary to remove the trypsin from the test solution after preincubation with this enzyme. As shown in the upper part of Fig. 2, trypsin having a molecular weight of 23,700 (13) did not sediment upon density gradient centrifugation into the first four (counted from the bottom) fractions. Addition of 0.4 ml of fractions 3 and 4 as test solutions to the assay system allowed base-line incorporation (188 cpm) of glycine-^{14}C into protein. Hence, the elimination of the "supernatant factor" in fraction 4 (lower part of Fig. 2) is ascribed to trypsin digestion during the preincubation. Preincubation with trypsin also inactivated the "supernatant factor" present in an equal weight of adrenals from control rats.

Preincubation with ribonuclease did not cause inactivation of the "superna-

tant factor." "Supernatant factor" from adrenals of (adrenocorticotrophin-gel, Upjohn, 140 USP units intramuscularly over 3 days)—treated rats was prepared by concentrating the activity from the 120 min $\times$105,000 $\times g$ supernatant into a loose pellet by centrifugation at 140,000 $\times g$ for five hours and was preincubated for 30 minutes at 37 C with and without 20 μg ribonuclease. The incubation mixtures were centrifuged for 15 hours at 37,500 rpm and +1 C on a 5–13% sucrose gradient, and aliquots of the fractions of both gradients were assayed for stimulation of glycine incorporation in the usual test system. Neither the total activity nor the pattern of sedimentation of the "supernatant factor" was affected by treatment with ribonuclease. This lack of effect was confirmed by experiments in which bentonite was used for the adsorption of ribonuclease.

In similar experiments the effect of 0.5% sodium lauryl sulfate on the glycine-^{14}C incorporation was studied. No effect of sodium lauryl sulfate (14) on the sedimentation of the activity on sucrose gradients was observed, suggesting that the material is not a nucleoprotein.

Phenol extraction (15) of either 105,000 $\times g$ supernatant of adrenals from rats treated with adrenocorticotrophin-gel or active fractions obtained after density gradient centrifugation resulted in complete loss of stimulation of glycine-^{14}C incorporation. Care had to be taken to assure complete extraction of protein from the aqueous layer, and, when necessary, the phenol treatment was repeated (14). Ribonucleic acid prepared by the procedure of Sevag *et al.* (16) was inactive when added to the assay system.

The above results suggested the protein nature of the "supernatant factor," and it was suspected that it might be a transfer enzyme. The dialyzed 105,000 $\times g$ supernatant of adrenals from rats treated with adrenocorticotrophin-gel was chromatographed on a diethylaminoethyl cellulose column as described by Takanami (17). The diethylaminoethyl cellulose was prepared as previously described (18). More than 90–95% of the recovered activity was eluted by the KCl gradient into samples 5 and 6 (Fig. 3), *i.e.*, between 0.15 and 0.275M potassium (approximately 0.17 and 0.295M chloride). The highest specific activity was in sample 6. The chromatography on diethylaminoethyl cellulose provided a total yield of 53, 58 and 70% of the stimulating activity. The pattern of elution was identical with the one described by Takanami (17) for transfer enzyme.

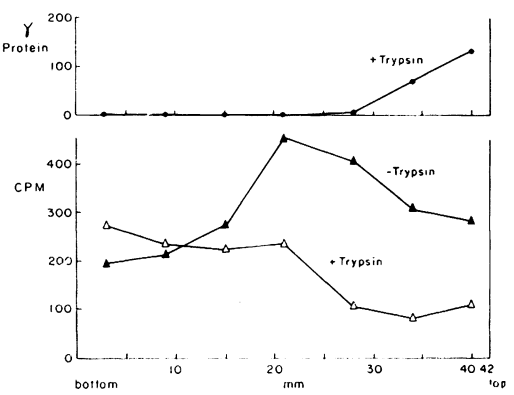

FIG. 2. Effect of trypsin on the factor stimulating glycine-^{14}C incorporation. The fractions of a density gradient (5–13% sucrose) containing the "supernatant factor" (*cf* Fig. 1) were combined and the active material was concentrated into a loose pellet by centrifugation for 5 hr at 140,000 $\times$ *g*. This material was incubated for 60 min at 37 C in the absence and in the presence of trypsin, 2 mg in 0.4 ml. The 2 incubation mixtures and an equivalent amount of trypsin were each layered on top of a 5–13% sucrose gradient. The 3 samples were centrifuged simultaneously for 16 hr at 37,500 rpm at +1 C. Triangles are glycine-^{14}C incorporated into protein by the assay system upon addition of 0.4 ml of each fraction of the density gradients which contained the "supernatant factor." Points are μg of protein per 0.1 ml of each fraction of the density gradient which contained trypsin alone.

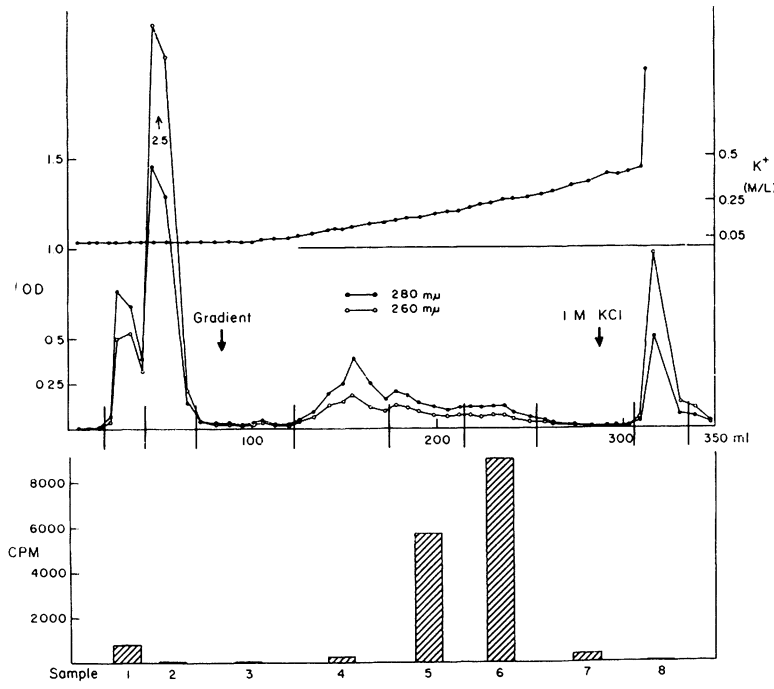

FIG. 3. Distribution of activity following chromatography on diethylaminoethyl cellulose of the 105,000 × *g* × 2 hr supernatant prepared from the homogenate of 1473 mg of adrenals from rats treated with adrenocorticotrophin-gel. After determination of the optical density at 260 and 280 mμ, the fractions collected from the column were recombined into 8 samples as indicated by the vertical bars. The activity of aliquots was determined by the test system, and the total stimulatory activity (columns) was calculated. Protein contents of these samples were: 1, 5.7 mg; 2, 1.1 mg; 3, 0.52 mg; 4, 4.98 mg; 5, 5.0 mg; 6, 3.36 mg; 7, 0.29 mg; 8, 0.38 mg.

The amino acid-activating enzymes are eluted before this fraction.[5]

Sucrose gradient centrifugation of the preparation with the highest specific activity (sample 6, Fig. 3) showed only partial sedimentation of the protein into the fraction where the "supernatant factor" moved, indicating further purification. Upon storage at 3 C, 50% of the activity in sample 5 (Fig. 3) was recovered after four weeks.

Aliquots of samples 5 and 6 (Fig. 3) were now assayed for their protein and RNA content. A preparation containing 2.5 mg protein and weighing 2.6 mg was dissolved in distilled water and precipitated with cold 5% TCA, washed with ethanol-ethyl-acetate, extracted twice for 15 minutes at 90 C with 5% TCA and washed again with ethanol. The dry weight of the residue was 2.7 mg and it contained 2.4 mg of protein. In both the cold and the hot trichloroacetic acid extracts, chemical analysis for RNA was carried out as described by Schneider (19), using the colorimetric assay for ribose of Dische (20). Zero readings were obtained. From the limits of sensitivity of this technique, this preparation contained less than 1% ribonucleic acid. The "supernatant factor" prepared by diethylaminoethyl cellulose chromatography was shown (Table 4) to stimulate the incorporation of both glycine-^{14}C and leucine-^{14}C into protein.

[5] Schatz, D., and W. J. Reddy (unpublished observations).

Discussion

In this study, the 15,000 × *g* supernatant fraction of pig adrenals was chosen as the cell-free protein-synthesizing system. It had the obvious advantage that the small amount of rat adrenal tissue was not a problem. On an equal weight basis, pig adrenal supernatant appears to be approximately

twice as responsive to the "supernatant factor" from rat adrenals as the control rat adrenal 15,000 $\times g$ supernatant (1).

The "supernatant factor" from rat adrenals was active in the 15,000 $\times g$ supernatant from rat, pig, dog and rabbit adrenals, whereas it did not stimulate glycine incorporation in an analogous preparation from normal rat liver, indicating organ rather than species specificity.

The effect observed with commercial preparations of adrenocorticotrophin could have been caused by impurities. That this effect was due to adrenocorticotrophin has been demonstrated in experiments with a synthetic peptide. Infusion[6] of this compound into hypophysectomized rats gave rise to material in the soluble fraction of the adrenals that stimulated protein synthesis in the cell-free system and sedimented on sucrose gradients exactly as the "supernatant factor" from the adrenals of rats treated with adrenocorticotrophin-gel. Although the synthetic peptide is only a portion (23 amino acids) of the "physiological" nonatriacontapeptide, the assumption that the observed effect is caused by the physiological principle of adrenocorticotrophin seems justified.

The factor stimulating amino acid incorporation has a molecular weight of approximately 1.0 to 1.4 $\times 10^5$ (S value = 6–7) and has the properties of a protein. Preincubation at 55 C or with trypsin destroyed the activity. It was not inactivated by preincubation with ribonuclease. Nucleic acid fractions prepared by phenol extraction, in which exhaustive extraction of protein material into the phenolic layers was assured, had no activity. Finally, more than 90% of the activity recovered after chromatography on diethylaminoethyl cellulose was shown to be of protein nature, with virtually no ribonucleic acid detectable upon chemical analysis.

TABLE 4. Incorporation of glycine-^{14}C and leucine-^{14}C into protein

Test solution	cpm in Protein	
	Glycine-^{14}C	Leucine-^{14}C
I	162	24
II	892	366
III	1331	648

The complete incubation mixtures (*cf* Methods) contained either 1 μc = 0.36 μmole glycine-^{14}C or leucine-^{14}C. Test solutions added were: I = medium A; II = 0.36 mg, and III = 0.72 mg of "supernatant factor" as prepared by diethylaminoethyl cellulose chromatography (*cf* Fig. 3) in 0.4 ml medium A. For incubation conditions and determination of the radioactivity *cf* Methods.

Recent evidence (21–23) indicates that, in addition to amino acid activating enzymes, s-RNA and ribosomes, two transfer enzymes are involved in protein synthesis. Earlier studies had located the stimulation of protein synthesis to a nondialyzable substance which acted beyond the formation of amino acyl transfer ribonucleic acid (1). The pattern of elution of the activity from the diethylaminoethyl cellulose columns was identical with the one observed by Takanami (17) upon preparation of transfer enzyme from rat liver. This and the protein nature of the "supernatant factor" suggest that it is probably a transfer enzyme.

Ferguson (25) has reported that actinomycin markedly inhibits adrenal ribonucleic acid synthesis but does not affect adrenal steroidogenesis, which suggests that adrenocorticotrophin does not act through synthesis of messenger ribonucleic acid. In previous work (24), puromycin was shown to block steroido-

[6] We wish to express our gratitude to Dr. Philip Hirsch, Harvard Dental School, for instructing us in the technique and for providing equipment used in the infusion experiments with hypophysectomized rats.

genesis, indicating a relationship between adrenocorticotrophin-induced steroidogenesis and protein synthesis. Since it has been shown previously (1) that there is no increased activity of amino acid-activating enzymes or transfer ribonucleic acids and messenger ribonucleic acid appears to be excluded by the studies of Ferguson, the only reasonable explanation left is that adrenocorticotrophin increases the activity of a transfer enzyme.

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