Adrenal Cortical Hormone and Incorporation of Radioactivity into Nucleic Acid of Isolated Rat Diaphragm.* (27571)

LLOYD A. FERGUSON AND IRA G. WOOL
Department of Physiology, University of Chicago, Chicago, Ill.

Adrenalectomy enhances, and adrenal cortical hormone depresses, the incorporation of C^{14} from amino acids, and from amino acid precursors, into the protein of isolated rat diaphragm(1,2,3). These changes are, at least in part, the result of a direct effect on protein biosynthesis(1,3), but neither the manner nor the exact site of corticosteroid action is known.

The several species of nucleic acids play a pre-eminent role in protein synthesis(4). Soluble ribonucleic acid (RNA) participates in transport of activated amino acids to the site of protein synthesis(5); ribonucleoprotein RNA acts at the site of synthesis in a manner still to be determined; finally, a specific unstable RNA, termed messenger RNA, is synthesized in the nucleus in a manner complimentary to chromosomal deoxyribonucleic acid (DNA) and serves to encode the ribonucleoprotein particle for synthesis of a specific protein(6). There is also evidence that for protein manufacture *de novo*, synthesis of at least a fraction of cellular RNA is, in some instances, obligatory(6). For these reasons we have investigated the effect of adrenalecomy and hydrocortisone on incorporation of C^{14} from labeled adenine into total nucleic acid and RNA of isolated rat diaphragm, having in mind the possibility that nucleic acid synthesis may be the intracellular process on which corticosteroids act to alter protein synthesis.

Material and methods. Animals. Male Sprague-Dawley rats were maintained under standard conditions(7) until they reached 110-130 g. The adrenal glands were removed under ether anesthesia and the animals were given 1% saline to drink; adrenalectomized rats were used on the 4th post-operative day. In the sham operations the adrenals were exposed but not injured. Hydrocortisone acetate in aqueous suspension was administered to intact rats in 2 doses (9:00 a.m. and 5:00 p.m.) of 1 mg each, except on the 4th day when the entire daily dose was given 4 hours before the diaphragms were removed.

Preparation of diaphragms. The rats were killed by decapitation and the hemi-diaphragms rapidly removed and prepared for incubation(7). Each hemi-diaphragm was incubated in 1 ml of Krebs-Henseleit bicarbonate buffer containing 1 μc of adenine-8-C^{14} sulfate hemihydrate (final concentration 2.7×10^{-4}M); the labeled adenine, purchased from Nuclear-Chicago Corp., had a specific activity of 5.06 mc/mM. Incubation was for 2 hours at 37° in a Dubnoff shaker (152 cycles/min); the gas phase was 95% oxygen–5% carbon dioxide. In the experiments in which the nucleic acid content of the diaphragm was determined (Table II) the hemi-diaphragms were removed, blotted lightly, weighed and without being incubated immediately treated as described below.

Incorporation of C^{14} into total nucleic acid, RNA and DNA. After incubation each hemi-diaphragm was placed in an iced mortar, minced, and ground to a slurry in sand with 10% cold trichloroacetic acid. In preliminary experiments the nucleic acid fraction was prepared by the method of Schneider(8); it was found, however, that separation of the nucleic acids and the adenine-8-C^{14} was not complete, significant radioactive contamination occurring in zero-time controls (average 8.5% in 3 experiments). For this reason it was decided to isolate specifically the sodium nucleates by the method first described by Barnum and Huseby(9) and later modified by Hecht and Potter(10). The ground diaphragm was washed 3 times with cold 10% trichloroacetic acid and 4 times with cold 95% ethanol, then extracted with 5 ml of 10% sodium chloride at 100° for 30 minutes. During the extraction the pH was kept at 7.0 by

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TABLE I. Effect of Adrenalectomy and Hydrocortisone on Incorporation of Radioactivity from Adenine-8-C^{14} into Total Nucleic Acid and RNA of Isolated Rat Diaphragm.

		Radioactivity incorporated			
		Nucleic acid c.p.m./O.D. unit (E_{260})		RNA c.p.m./O.D. unit (E_{650})	
I	Normal	293 ± 30.9	P <.001	355 ± 12.8	P <.001
	Adrenalectomized	551 ± 39.1		799 ± 57.7	
	Hydrocortisone, 2 mg/day	281 ± 9.4		371 ± 51.3	
II	Normal	261 ± 19.5		380 ± 27.0	
	Sham operated	275 ± 25.6	P <.001	370 ± 18.5	P <.01
	Adrenalectomized	458 ± 29.9		608 ± 42.5	

Each figure is the mean ± S.E. of 6 observations. Value of P for a difference which is significant is indicated.

addition of sodium hydroxide with phenol red as an indicator. The residue was reextracted and sodium nucleates were precipitated from the combined sodium chloride extracts with 2.5 volumes of 96% ethanol at −10° overnight. The precipitate was collected by centrifugation, washed twice with cold 96% ethanol, and dissolved in 3 ml of 0.1 N sodium hydroxide; a 0.5-ml aliquot was placed on a stainless steel planchet with concentric rings and counted with a glass flow Geiger counter (Nuclear-Chicago Corp., model D-47) having an efficiency of 40%. No radioactivity was found in zero-time controls prepared by this method. A second 0.5-ml aliquot was diluted with 2.5 ml of water and nucleic acid content determined by the extinction at 260 mμ in the Beckman model DU spectrophotometer; the results are expressed as counts per minute per optical density unit at 260 mμ. The remaining 2 ml of the original sample (in 0.1 N sodium hydroxide) was incubated for 24 hours at 37°(11). The DNA was precipitated with 0.1 volume of 2 N hydrochloric acid and washed once with 1 ml of 0.2 N hydrochloric acid. RNA content of the pooled supernatant fractions was determined by the orcinol method(12), DNA by the indole procedure(13). The results are expressed as optical density units per g of diaphragm (wet weight). Specific activity of the RNA fraction was usually calculated by using the radioactivity of the total nucleic acid fraction since little incorporation of radioactivity into DNA occurred. The validity of this method was determined by measuring the radioactivity of the RNA fraction directly; essentially the same results were obtained. Incorporation of radioactivity into RNA is expressed as counts per minute per optical density unit at 650 mμ.

Results. Adrenalectomy enhanced the incorporation of C^{14} from adenine into the sodium nucleates and into the RNA isolated from rat diaphragm; hydrocortisone (2 mg/day) administered to intact rats was without effect (Table I). Since sham operation did not alter incorporation of radioactivity into nucleic acid, the increase after adrenalectomy must be a result of the deprivation of adrenal steroids.

Adrenalectomy also increased the amount of nucleic acid and of RNA present in diaphragm; once again hydrocortisone caused no change (Table II).

An attempt was made to assess the effect of adrenalectomy and of hydrocortisone on incorporation of radioactivity from C^{14}-adenine into the DNA of diaphragm. Under the conditions of our experiments, insufficient counts were incorporated (less than 2% of the amount in RNA) to permit a conclusion concerning the effect of adrenal steroids on DNA synthesis.

Discussion. Adrenalectomy enhances the synthesis of skeletal muscle RNA as evinced by: (1) an increase in incorporation of C^{14} from adenine into rat diaphragm incubated *in vitro;* and (2) by the total amount of RNA extractable from diaphragm. The increase in RNA synthesis parallels the increase in incorporation into skeletal muscle protein of radioactivity from amino acids(1,2) and from amino acid precursors(3). Since the several intracellular molecular species of RNA are essential to protein synthesis, these findings

TABLE II. Effect of Adrenalectomy and Hydrocortisone on Total Nucleic Acid and RNA Content of Rat Diaphragm.

Exp	Condition	Nucleic acid O.D. units (E_{260})/g of diaphragm (wet wt)		RNA O.D. units (E_{650})/g of diaphragm (wet wt)	
I	Normal	19.4 ± .91	P <.02	19.5 ± 1.45	P <.001
	Adrenalectomy	29.8 ± 3.52		25.8 ± .32	
	Hydrocortisone, 2 mg/day	18.5 ± 1.37		17.3 ± 1.34	
II	Normal	18.0 ± 1.10			
	Sham operated	19.1 ± .96	P <.001		
	Adrenalectomy	25.8 ± .74			

Each figure is mean ± S.E. of 6 observations. Value of P for a difference which is significant is indicated.

are not entirely unexpected. Indeed, it would have been more surprising if the increased protein synthesis observed after adrenalectomy had not been associated with a similar alteration in RNA metabolism. It is tempting to conclude that RNA synthesis is the intracellular locus at which adrenalectomy acts to increase protein synthesis. There is, of course, no assurance that this is the case. It is at least equally possible that the effect of adrenalectomy on protein and RNA synthesis stems from a single and more primary action at some other site, as for example the more efficient utilization and/or coupling of energy for anabolic processes.

Hydrocortisone administration was without effect on RNA synthesis, despite the fact that the hormone consistently produces a striking decrease in the incorporation of amino acids into protein(1,2). Under these circumstances it is unlikely that an alteration in RNA synthesis or in RNA turnover is the means by which adrenal cortical hormone inhibits protein biosynthesis. This is not the first observation that the influence of adrenalectomy, and of cortisone treatment of intact rats, are not coextensive. Corticosteroids decrease the accumulation of amino acids by isolated rat diaphragm while adrenalectomy is without effect on amino acid transport(14, 15). The influence of adrenalectomy on protein biosynthesis is best viewed as resulting from withdrawal of an optimal amount of adrenal steroids and not necessarily as existing on a continuum with the effect of administration of large amounts of adrenal cortical hormone to intact rats.

There are difficulties in interpretation of results obtained in experiments where the incorporation of precursors into nucleic acids has been measured. Among the variables difficult of control is the size of the intracellular precursor pool, of each of the intermediates between the precursor and the nucleic acids, and of the nucleic acids themselves; each will influence the extent to which the particular precursor appears in nucleic acids. We determined only total RNA. Hence the conclusions drawn concerning hormone effects are not exclusive; further information is necessary concerning compartment sizes and synthetic pathways.

Isolation of the primary biochemical locus at which adrenalectomy influences metabolic processes is important since it is vital to an analysis of the mechanism of action of adrenal steroids. Especially pertinent in this context is identification of the exact molecular species of RNA whose synthesis is increased by adrenalectomy (if indeed it has that specific an affect). An effect on production of a specific RNA, especially if that RNA were limiting for protein synthesis as may be the case for messenger RNA(6), would support the speculation that RNA synthesis is the intracellular site of alteration of protein synthesis after adrenalectomy.

Summary. Adrenalectomy stimulated incorporation of radioactivity from adenine-8-C^{14} into total nucleic acid and the RNA of isolated rat diaphragm. Moreover, adrenalectomy increased the actual amount of nucleic acid and of RNA that could be extracted from rat diaphragm. Cortisone administration (2 mg/day) was without effect on incorporation of C^{14} from adenine into nucleic

acid or RNA, nor did it influence their amount.

1. Wool, I. G., Weinshelbaum, E. I., *Am. J. Physiol.*, 1959, v197, 1089.
2. Manchester, K. L., Randle, P. J., Young, F. G., *J. Endocrinol.*, 1959, v18, 395.
3. Wool, I. G., Weinshelbaum, E. I., *Am. J. Physiol.*, 1960, v198, 360.
4. Berg, P., *Ann. Rev. Biochem.*, 1961, v30, 293.
5. Hoagland, M. B., Zamecnik, P. C., Simpson, M. L., *Biochem. et Biophys. Acta*, 1957, v24, 215.
6. Brenner, S., Jacob F., Meselson, M., *Nature*, 1961, v190, 576.
7. Wool, I. G., Krahl, M. E., *Am. J. Physiol.*, 1959, v196, 691.
8. Schneider, W. C., *Methods in Enzymology*, Acad. Press, New York, 1957, v3, 680.
9. Barnum, C. P., Huseby, R. A., *Arch. Biochem.*, 1950, v29, 7.
10. Hecht, L. I., Potter, V. R., *Cancer Research*, 1956, v16, 988.
11. Schmidt, G., Thannhauser, S. J., *J. Biol. Chem.*, 1945, v161, 83.
12. Mejbaum, W., *Z. Physiol. Chem.*, 1939, v258, 117.
13. Ceriotti, G., *J. Biol. Chem.*, 1952, v198, 297.
14. Wool, I. G., *Nature*, 1960, v186, 728.
15. ——, *Am. J. Physiol.*, 1960, v199, 715.

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Simplified Specimen Preparation for Electron Microscopic Studies of an Intra-erythrocytic Parasite, *Anaplasma marginale*. (27572)

A. E. RITCHIE (Introduced by Martin H. Roepke)
National Animal Disease Laboratory, Animal Disease and Parasite Research Division, Agricultural Research Service, U. S. Department of Agriculture, Ames, Iowa

Hemoglobin and the cytoplasmic matrix of the intact bovine erythrocyte preclude direct electron optical observation of the morphology of its associated intra-cellular parasite, *Anaplasma marginale*.

Various methods of dehemoglobinization of *anaplasma* infected erythrocytes have been previously reported. These include freezing and thawing(1), distilled water lysis of blood smears(2), distilled water lysis of washed and packed cells(3), and lysis induced by saponin(1) or specific hemolysin(1). Transfer of the hemolyzed stromata to specimen grids has been effected by several technics: pseudo-replication from glass(2) or from agar(1), nebulizing(3), collodian stripping of a positive replica prepared by pressure molding polyethylene(3), and evaporation of a droplet of the stroma suspension directly onto a filmed grid(1). In most instances the final preparations were lightly shadowed with metal prior to microscopy.

For routine electron microscopy of this intra-erythrocytic parasite the above methods involved one or more of the following disadvantages: (1) lengthy or tedious procedure, (2) obscuring of morphological detail by addition of extraneous mass to the specimen, (3) derangement or alteration of anatomical structures as a result of an applied physical stress, and (4) separation of the organism from its host cell stroma.

Attempts were made in the present study to hemolyze *anaplasma* infected bovine erythrocytes and to prepare specimen grids under conditions which would retain sufficient structural integrity of the parasite to facilitate its recognition directly within the erythrocyte stroma.

Materials and methods. Blood samples from splenectomized calves in the acute phase of the disease were collected in tubes containing heparin.

Dehemoglobinization. Preparation of the bovine erythrocyte stromata containing the parasites was carried out by a dialysis procedure similar to that devised by Danon, Nevo and Marikovsky(4). These workers have shown that human stromata prepared by dialyzing an erythrocyte suspension against hypotonic solution consisted of thin membranes with a granular surface structure and were free of rents, "craters" or ruptures. Two or 3 drops of infected whole blood were added