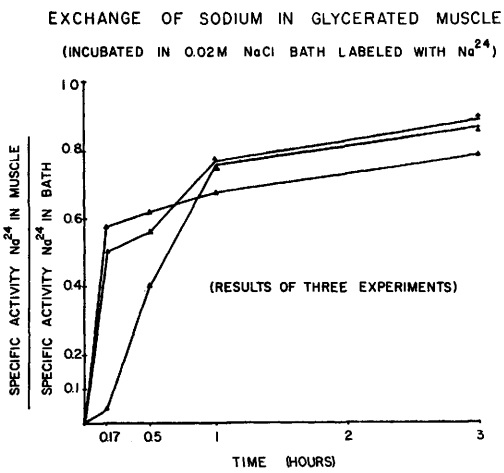

FIG. 2. Results of studies of Na^{24} exchange with sodium in glycerated muscle. All points are avg of duplicate determinations.

as indicating that there are probably at least 2 compartments in the system, a rapidly exchanging and a slowly exchanging compartment.

Conclusions. 1. Rat skeletal muscle is found to contain sodium after extraction with 50% glycerol-water followed by 0.08 M KCl. There was 1.24 meq/kg wet weight in a typical series. Sodium was present after further extraction with distilled water. Average Na content of 16 samples so extracted was 0.84 meq/kg. 2. Electronmicroscopic studies reveal intact nuclei and collapsed mitochondria as possible sites of this sodium. 3. The sodium present after glycerine and KCl extraction exchanges with Na^{24}. The rate is not a simple first order reaction, and suggests that the sodium exists in at least two compartments. 4. A small portion of the sodium appears to be in a compound which is soluble in ether. 5. Evidence is presented that this sodium is not associated with the matrix of the connective tissue in muscle.

Summary. The premise that intracellular sodium is functionally a simple solution in a cell membrane is questioned by the authors and others(2,9). The data reported are interpreted as indicating that there is a component of intracellular sodium which is characterized by not being exchangeable with potassium ions. Fenn felt that such sodium ions were bound to myosin(1). The nuclei and mitochondria are suggested as additional possible sites of binding of such sodium.

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1. Fenn, W. O., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 783.
2. Craig, A. B., Jr., Ohr, E. A., Fenn, W. O., *Am. J. Physiol.*, 1961, v200, 561.
3. Dalton, A. J., *Anat. Rec.*, 1955, v121, 281.
4. Audia, M., *Am. J. Physiol.*, 1958, v195, 702.
5. Levitt, M. F., Turner, L. B., Sweet, A. Y., Pandiri, D., *J. Clin. Invest.*, 1956, v35, 98.
6. Muntwyler, E., Mellors, R. C., Mautz, F. R., Mangun, G. H., *J. Biol. Chem.*, 1940, v134, 389.
7. Manery, J. F., Danielson, I. S., Hastings, A. B., *ibid.*, 1938, v124, 359.
8. Harris, E. J., Steinbach, H. B., *J. Physiol.*, 1956, v133, 385.
9. Conway, E. J., *Physiol. Rev.*, 1957, v84, 37.

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Latex Agglutination Reactions Between Human Chorionic Gonadotropin and Rabbit Antibody.* (27190)

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The antigenicity of the partially purified protein hormone, human chorionic gonadotropin (HCG), in the rabbit has been reported(1). This rabbit antibody has been employed in precipitin and in hemagglutination-inhibition technics as a serologic test for pregnancy(2,3).

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Polystyrene latex particles have been used as agglutinable carriers in a variety of antigen-antibody systems (*e.g.*, 4,5,6). The latex particle carrier apparently operates by strong adsorption of the initial protein added(7).

The purpose of the present report is to show first, that this antigen-antibody system is amenable to use of polystyrene latex particles in an agglutination reaction; second, that the reaction may be used to detect rabbit antibody by employing hormone-coated particles, or conversely, that the hormone may be measured by employing antibody-coated particles; and third, that the rabbit antibody is specifically neutralized by the hormone antigen.

Materials and methods. Production of antibody. Young adult male and female white rabbits were injected intramuscularly at weekly intervals for 4 weeks with 10,000 International Units (I.U.) of Human Chorionic Gonadotropin (HCG) (Antuitrin S†) in normal saline and emulsified in a like volume of a Freund's type adjuvant. The adjuvant was prepared as follows: 1 g of Lanum (Lanolin, Merck) was dissolved by heating with 9 ml of Whitemore Oil No. 2 (Consolidated Laboratories); to this was added 4 mg of heat-killed *Mycobacterium phlei*. To 1 ml of this suspension was added 1 ml of the saline solution of HCG containing 10,000 I.U. of hormone and the substances were thoroughly emulsified. Two weeks after the last intramuscular injection the rabbits were given 500 I.U. of the hormone in saline intradermally twice weekly for 2 weeks; the animals were rested for 2 weeks and then bled by cardiac puncture. The separated sera were preserved by adding Merthiolate to a concentration of 1:10,000.

Polystyrene latex. Bacto-Latex (Difco), average diameter of particles, 0.81 μ, was used.

Phosphate-buffered saline. Each liter contained: 1.31 g KH_2PO_4, 5.75 g Na_2HPO_4, and 2.83 g NaCl. This solution was adjusted to pH 7.4 by addition of small amounts of either of the buffer salts.

† The Antuitrin S, derived from urine of pregnant women, was kindly supplied by Parke, Davis & Co., Detroit, Mich.

Test of rabbit antibody. This was the standard agglutination procedure in which dilutions of antibody were allowed to react with a constant concentration of antigen. A solution of HCG in saline was diluted in the phosphate buffered saline (PBS) so that final concentration of the hormone was 25 I.U./ml. To this solution was added latex, from the stock suspension, to a final dilution of 1:30. This mixture was allowed to stand at room temperature overnight. The rabbit sera were diluted 2-fold serially through 9 tubes in 0.2 ml amounts of PBS. The tenth tube (control) contained 0.2 ml of PBS. To each tube was added 0.1 ml of hormone-coated latex particles; after mixing, the tubes were incubated for 1 hour at 37°C, 1 hour at 5°C, and finally for 1 hour at 56°C. All tubes were then centrifuged at high speed for 5 minutes and observed for agglutination by reading over a slit lamp.

Test for human chorionic gonadotropin. Initial tests yielded bizarre zoning results which were eliminated by treating the antibody-containing sera to remove most of the albumin. Control and normal rabbit sera were similarly treated. The globulins were precipitated and separated from the albumin by use of the method of Deutsch(8). Precipitate A, containing all the globulins, was redissolved in PBS and 0.2 ml of this solution was serially diluted in 0.2 ml amounts of PBS through 9 tubes while the tenth tube (control) contained PBS only. To each tube was added 0.1 ml of 1:30 latex in PBS. After mixing, the tubes were allowed to stand for 1 hour at room temperature. Prolonged incubation resulted in spontaneous nonspecific agglutination. At the end of incubation time different concentrations of the hormone in PBS were added, and the tubes were then incubated and read as before.

Neutralization of rabbit antibody by the antigen. Normal and immune rabbit sera were diluted 2-fold in PBS in 0.2 ml amounts through 9 tubes; the tenth tube contained PBS only. This process was repeated through 6 rows of tubes for each serum. To the tubes of each row was added 0.2 ml amounts of different concentrations of the hormone in PBS. The antigen and antibody

TABLE I. Latex Agglutination Test for Rabbit Anti-Human Chorionic Gonadotropin with Hormone-Coated Latex Particles.

Rabbit No.	Dilution of rabbit serum 1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	Control
5	3*	4	4	4	4	4	3	2	1	—
6	3	4	4	3	2	2	1	1	1	—
7	3	4	4	3	3	2	1	1	1	—
8	3	4	3	3	3	3	3	2	2	—
Normal rabbit	—	—	—	—	—	—	—	—	—	—

* Agglutination was read as follows: (—) no visible agglutination; (±) rough in appearance but not definitely agglutinated; (1) definite agglutination with small agglutinate size; (2) definite agglutination of intermediate size; (3) large agglutinates with a turbid background; (4) large agglutinates with a clear background.

mixtures were allowed to react for one hour at room temperature. At the end of this time 0.1 ml of the hormone-coated latex suspension, prepared as previously described, was added and all the tubes were incubated, centrifuged and observed for agglutination.

Results. Agglutination test between rabbit anti-HCG and HCG-treated latex particles. Whole rabbit serum, either individual or pooled, and rabbit globulin fraction A diluted and tested as described under "Test for rabbit antibody" with 0.1 ml of 1:30 latex in PBS containing 25 I.U. per ml of HCG, yielded the results shown in Tables I and II. All immunized rabbits produced antibody.

Table II also presents a comparison of whole and fractionated sera. It appears that the overt titer of antibody is increased by the serum fractionation which decreases albumin content.

Latex agglutination test for human chorionic gonadotropin using latex particles coated with anti-HCG. Serial 2-fold dilutions of normal and immune rabbit globulin in 0.2 ml amounts of PBS were incubated for one hour with 0.1 ml amounts of the stock latex suspension diluted 1:30 in PBS. Various dilutions of hormone in 0.2 ml volumes in the same buffer were added and all tubes were incubated, centrifuged and read as before.

TABLE II. Latex Agglutination Titers and Total Proteins on Pooled Whole and Fractionated Rabbit Sera.

Serum	Titer	Total protein,* g %
Pooled normal rabbit serum	Negative	6.3
" immune " "	1:32 (4+)	6.7
Pooled normal rabbit precipitate A	Negative	4.3
Pooled immune rabbit precipitate A	1:256 (4+)	5.2

* Biuret method.

The data given in Table III show that the rabbit antibody globulin coated to the latex particles for one hour at room temperature renders the agglutination system capable of detecting the presence of the hormone in solution. It is seen also that the varying antigen concentrations exhibit a definite prozone reaction in the region of antigen excess. The sensitivity of detection of hormone by the immune rabbit globulin-coated latex particles appears much lower than when testing for antibody using latex coated with the hormone at a concentration of 25 I.U./ml (Table II).

Latex agglutination test for inhibition of the rabbit antibody by the hormone (antigen). Normal and immune rabbit serum was diluted 2-fold in PBS in 0.2 ml amounts; 6 sets of tubes were prepared. To each row of tubes was added one of the series of concentrations of the hormone in 0.2 ml of PBS. The tenth tubes and the sixth row were controls containing no antibody or antigen. The tubes were incubated at room temperature for one hour after which 0.1 ml of the 1:30 diluted stock latex particles coated with the hormone as before in a concentration of 25 I.U./ml was added. After mixing, the tubes were incubated, centrifuged and agglutination of the latex recorded.

Table IV shows that activity of the antibody is inhibited by the increasing amounts of soluble hormone added prior to addition of latex bound hormone. These results further suggest that the antibody is specific for

TABLE III. Latex Agglutination Test for Human Chorionic Gonadotropin with Antibody Globulin-Coated Latex Particles.

	HCG conc., I.U./ml	Dilution of globulins (precipitate A) added to latex 1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	Control
Normal rabbit globulins	0	—*	—	—	—	—	—	—	—	—	—
	12.5	—	—	—	—	—	—	—	—	—	—
	25	—	—	—	—	—	—	—	—	—	—
	50	—	—	—	—	—	—	—	—	—	—
	100	—	—	—	—	—	—	—	—	—	—
	200	—	—	—	—	—	—	—	—	—	—
Immune rabbit globulins	0	±	—	—	1	1	1	±	—	—	—
	12.5	4	4	4	4	—	—	—	—	—	—
	25	4	4	4	2	2	—	—	—	—	—
	50	4	3	3	2	1	—	—	—	—	—
	100	2	2	—	—	—	—	—	—	—	—
	200	1	—	—	—	—	—	—	—	—	—

* Reactions read as in Table I.

the antigen against which the rabbits were immunized, *i.e.*, Human Chorionic Gonadotropin.

Discussion. A direct test for Human Chorionic Gonadotropin, using latex particles coated with hormone as antigen, is readily performed with immune rabbit serum (Tables I, II). However, this procedure failed when the converse situation was used, *i.e.*, to react rabbit serum antibody-coated latex particles with hormone in solution. When the antibody-containing rabbit serum was treated to rid it of most, but not all, of the albumin fraction, this second procedure was found to be operative (Table III).

Wide and Gemzell(3) have shown that an agglutination inhibition system is suitable as a test for presence of the hormone using tanned sheep red blood cells with adsorbed hormone. It has been shown here (Table IV) that this same procedure operates with the polystyrene latex system.

We have chosen to study characteristics of this serologic reaction when latex is employed as the carrier of either antigen or antibody for practical reasons. That is, the system seems sensitive enough to detect concentrations of hormone which are detected by *in vivo* tests of pregnancy. However, detection of partially purified hormone in aqueous solution has proved to be less a problem than detection of hormone in urine or serum. Effort to establish the technic of a suitably sensitive and specific test of HCG in its normal menstruum is being actively explored.

Summary. It has been shown that rabbit antibody may be formed to the antigen, Human Chorionic Gonadotropin (Antuitrin S). Polystyrene latex particles were found to serve as an agglutinable carrier for this anti-

TABLE IV. Latex Agglutination-Inhibition Test for Human Chorionic Gonadotropin. Antibody plus hormone solution reacted with hormone-coated latex particles.

	HCG conc., I.U./ml	Dilution of serum 1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	Control
Normal rabbit serum	0	—*	—	—	—	—	—	—	—	—	—
	12.5	—	—	—	—	—	—	—	—	—	—
	25	—	—	—	—	—	—	—	—	—	—
	50	—	—	—	—	—	—	—	—	—	—
	100	—	—	—	—	—	—	—	—	—	—
	200	—	—	—	—	—	—	—	—	—	—
Immune rabbit serum	0	3	3	3	3	2	1	—	—	—	—
	12.5	3	3	3	1	—	—	—	—	—	—
	25	3	3	1	—	—	—	—	—	—	—
	50	2	±	—	—	—	—	—	—	—	—
	100	1	±	—	—	—	—	—	—	—	—
	200	—	—	—	—	—	—	—	—	—	—

* Reactions read as in Table I.

gen-antibody system. The possibility of using this agglutination test for HCG as a suitable test for pregnancy is being explored.

1. Rao, S. S., Shehani, S. K., *Immunol.*, 1961, v4, 1.

2. McKean, C. M., *Am. J. Obst. & Gyn.*, 1960, v80, 596.

3. Wide, L., Gemzell, C. A., *Acta Endocrinol.*, 1960, v35, 261.

4. Singer, J. M., Plotz, C. M., *Arthritis & Rheumatism*, 1958, v1, 142.

5. Saslaw, S., Carlisle, H. N., PROC. SOC. EXP. BIOL. AND MED., 1958, v97, 700.

6. Keele, D. J., Webster, J., *ibid.*, 1961, v106, 168.

7. Oreskes, I., Singer, J., *J. Immunol.*, 1961, v86, 338.

8. Deutsch, H. F., in *Methods in Medical Research*, 1952, v5, Year Book Publishers, Chicago, p. 284.

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Immunochemical Studies of Human Urinary Erythropoietin.* (27191)

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The presence of substances capable of specifically stimulating erythropoiesis has been demonstrated in plasma and urine of anemic animals and of humans with various hematologic disorders. Ion exchange procedures for fractionation and purification of erythropoietin obtained from the plasma of anemic sheep have been described and studies of the biochemical and physical properties of the various fractions have been made(1,2). The available evidence suggests that the active molecule in human urinary concentrates is either a mucoprotein or is intimately associated with a mucoprotein(3). It is not known whether the erythropoietically active material found in human urine is identical to the active material of anemic animal plasma. In general, no species specificity has been found. Concentration of the active material in human urine has been accomplished by ultrafiltration(4) and adsorption on kaolin(5). The present paper concerns an immunochemical study of erythropoietically active concentrates of human urine.

Materials and methods. Erythropoietically active urine obtained from anemic patients was concentrated by positive pressure ultrafiltration through collodion membranes as described by Van Dyke, Garcia, and Lawrence(4). Similar concentrates hereafter termed N were made of non-erythropoietically active human urine. The concentrate from an anemic patient has been termed E^{BS}. Comparison in the starved rat assay of the erythropoietic activity of these urinary concentrates with purified sheep erythropoietin obtained from Armour (Lot No. K 103-124) indicated that E^{BS} contains approximately 16 cobalt units/mg.

Three rabbits were immunized against E^{BS} by monthly injection of 16.6 mg of the urinary concentrate dissolved in 1 ml saline. Blood was collected from the central artery of the ear 6 days after the fourth injection; the serum was separated, pooled, and frozen until used. This serum has been termed anti-E^{BS}. Three rabbits were immunized against N by weekly injection of 1 mg of the concentrate dissolved in 1 ml saline. Serum termed anti-N was taken from these rabbits 6 days after the ninth injection. The first injections of E^{BS} and N were given subcutaneously (0.25 ml in each leg) after mixing with an equal volume of complete Freund's adjuvant (Difco); all subsequent injections were given intravenously.

Qualitative comparisons of the antigens in the urinary concentrates and the precipitins produced in rabbits by injection of these concentrates were made, using the technic of double diffusion in 2 dimensions developed by Ouchterlony(6). A solution of 0.5 or 1.0% agar containing 1% sodium azide was poured into petri dishes to obtain a layer about 6 mm

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