hemostat applied. Tracing the vagus cephalad by pulling caudad on the hemostat, the division of the right vagus is found slightly inferior to the level of the hilus of the right lung. Consequently, the hemostat is on the posterior branch of the right vagus. By pulling cephalad on the posterior right vagus and following it along the lateral wall of the esophagus, the junction of this branch with the posterior branch of the left vagus is easily located slightly above the diaphragm. Here, the left vagus has just emerged from behind the esophagus. Maintaining cephalad traction on the hemostat, two more hemostats are applied distal to the juncture and approximately 1½ to 3½ cm of the posterior vagus, formed by this union, resected. The cut ends are ligated with cotton.

Keeping in the mediastinum and crossing the esophagus anteriorly, the left vagus is easily identified running parallel to this structure invested with mediastinal pleura. After hooking the nerve, it is dissected partially free and a hemostat applied. By creating caudad traction on the hemostat, the junction of the anterior branches of the vagus nerves is brought into view. This union is below the bifurcation of the left vagus. The hemostat, therefore, is on the *anterior vagus*. Another hemostat is applied and 1½ to 3½ cm or more of the nerve removed, the cut ends also being ligated.

Thus, both vagus nerves have been disrupted in the thorax and the operation is complete except for wound closure.

Summary. 1. The thoracic vagus as seen in 42 dogs that came to autopsy is described.

2. A method is presented for its disruption.

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Studies on Antigens of Human Red Cells. II. Serological Properties of Rh Factor in Elinin.* (17771)

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In a previous report(1) a lipoprotein fraction of red cells containing the Rh and A and B factors was described and given the name elinin. A new term was required since it was shown that the post-hemolytic residue of red cells (stroma) could be separated into two fractions by solubility differences and that elinin was biologically distinct from S protein (stromatin) in that the latter did not contain Rh or A and B activity. Since it has not been possible to obtain a further concentration of the Rh factor in elinin by chemical methods, purification of elinin by physical methods was necessary in order to characterize its chemical and biological properties and to prepare a uniform substrate for further attempts at fractionation. The details of the steps taken in the purification of elinin and the analysis of its chemical and physical properties are reported elsewhere(2,3). When uniform solutions of elinin‡ became available for animal and human injection we hoped to obtain the answer to two questions: (1)

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1. Calvin, M., Evans, R. S., Behrendt, V., and Calvin, G., PROC. SOC. EXP. BIOL. AND MED., 1946, v61, 416.

2. Moscowitz, M., Calvin, M., and Evans, R. S., in press.

3. Danlicker, W. S., Moskowitz, M., Zimm, B., Calvin, M., in press.

‡ We have retained the name elinin for the purified fraction of red cells containing the Rh and A and B factors.

TABLE I.
Comparable Results in Inhibition Studies Using Anti-Rh_0 Serum with a Saline Agglutinin and a Serum with a Univalent or Blocking Antibody. Both sera showed an agglutinating titer of 1-64 in saline and serum dilutions.

Anti-Rh_0 saline agglutinating sera	Saline diluent					
Dilutions of Elinin	1–2	1–4	1–8	1–16	1–32	1–64
Elinin Rh_0 cells	0	0	0	++	+++	+++
" " " + 56°C	+++	+++	+++	+++	+++	+++
" Rh negative cells	+++	+++	+++	+++	+++	+++
Anti-Rh_0 univalent sera	Serum diluent					
Elinin Rh_0 cells	0	0	0	+	++	+++
" " " + 56°C	+++	+++	++	++	+++	+++
" Rh negative cells	+++	+++	+++	+++	+++	+++

Would elinin containing Rh activity prove antigenic in human subjects and (2) would it be possible to demonstrate neutralization of Rh antibodies *in vivo* in human subjects. While there is some evidence that the Rh factor in elinin is not antigenic when compared to whole red cells, we have as yet no evidence to indicate that injections of elinin in the amounts used so far will neutralize antibodies *in vivo*.

Methods and materials. We have continued to use the serum inhibition technic as the most reliable method of assay for the Rh factor in fractions from Rh-positive cells.

1. Assay of Rh activity in elinin; Anti-Rh' and anti-Rh_0 saline agglutinating sera and anti-Rh_0 univalent sera have been used with satisfactory results in the serum inhibition test. For serum inhibition tests the anti-Rh sera was diluted so as to show an agglutinating titer of 1-64 on further dilution, with a 2+ micro-agglutination of Rh positive cells at this dilution. The basic technic of the serum inhibition test has been described previously(1). Serial dilutions of antigen, which in this case is a 1% solution of elinin from Rh positive cells, were incubated with a constant strength of anti-Rh serum. The highest dilution of the elinin showing complete neutralization of the antiserum is referred to as the titer. If serum with a saline agglutinating antibody was used, saline was used as the diluent for the elinin, whereas normal human serum was employed if a univalent anti-Rh_0 antibody was used. The two types of sera gave comparable results with the same preparations of elinin as shown in Table I. Fresh Rh positive cells of known specificity and avidity were added to the serum elinin mixture to demonstrate the presence of unabsorbed antibodies. Elinin from homozygous Rh negative cells (cde/cde) was used as a control in some observations. The usual control, however, was elinin heated to 56° for 5 minutes which destroys the Rh activity.

2. Preparation of elinin for injection. Since elinin could not be produced by sterile technic, it was necessary to filter the final preparation before use. A 1% solution of elinin in distilled water was found to pass quantitatively through a fritted glass filter, (ultrafine, Corning Glass Works) without loss of Rh activity. The addition of electrolytes prior to filtration produced aggregation of molecules and impaired filtration. After filtration sterility was tested and the preparation was made isotonic by addition of electrolytes. Elinin proved to be pyrogenic when injected intravenously into animal and human subjects, so attempts were made to reduce contamination with pyrogenic materials and to remove pyrogens present. It has not been possible to develop a sterile technic for the preparation of elinin in the supercentrifuge and ultracentrifuge, but contamination has been reduced to a minimum. Only freshly distilled water was used in the process of hemolyzing the cells and suspending the elinin. An attempt was made to reduce the pyrogenicity of elinin by the use of "decalso," a cationic exchange agent, following the procedure of Smith and Pennel(4), but

4. Smith, W. E., and Pennell, R. B., *J. Bact.*, 1947, v54, 715.

this method was not effective. However, the following treatment appeared to reduce to some extent the pyrogenicity of elinin for rabbits. An acetate buffer with an ionic strength of 1 (100 ml M sodium acetate plus 50 ml M acetic acid) was added to a sterile 1% solution of elinin in the ratio of 2 ml of buffer per 100 ml elinin solution. The pH of the buffer when diluted with water in this ratio was 5 at 20°C. Sterile technic was used in this procedure and the pH of the elinin after the addition of the buffer was not taken. As the elinin was kept in an ice bath and centrifuged at 0°C, the pH was probably slightly greater than 5. The elinin precipitated after adding the buffer and was allowed to stand 15 minutes. It was centrifuged at 2000 G for 5 minutes and the colorless water-clear supernatant fluid was removed by suction with a capillary pipette. Phosphate buffer (μ = 0.15; pH 7.7 at 25°C) was added to the sediment to make the volume about equal to that of the original elinin solution. The sediment was mixed with the buffer by alternately drawing it into and expelling it from a pipette. It was allowed to stand 15 minutes, mixed again, and centrifuged at 2000 G for 15 minutes. The supernatant fluid was removed as above. The precipitated elinin was then ready to be injected. Four ml of this material in a concentration of 1% were injected into two rabbits; one had a rise in temperature of 0.5°C, the other manifested no change in temperature. Two ml in a concentration of 1% were injected intravenously into a normal female who was not sensitized to the Rh factor. She manifested a 0.5°C rise in temperature and complained of dizzy sensations 2 hours later. Four ml of the same material were injected into a male subject who had been sensitized to the Rh_0 antigen years previously by repeated transfusions. He developed a shaking chill, weakness, and his temperature rose to 39°C. This appeared to one of us a typical reaction to pyrogenic substances, and there was no evidence, such as asthmatic breathing or urticaria, that specific hypersensitivity was involved. As a result of the above reactions, it was decided to abandon the attempt to give large amounts of elinin intravenously to human subjects and to use the material available for intramuscular injection in approximately 8% concentration, which has not produced systemic reactions and has caused only transient local soreness.

Injection of elinin into animals and human subjects. Initial attempts to produce Rh antibodies by the injection of crude fractions of Rh-positive cells into rabbits and guinea pigs were not successful. Some of the 10 rabbits which received I.V. injections developed high titers of nonspecific red cell agglutinins (1-10,000), but none of the sera showed any specificity for Rh positive cells, either before or after adsorption with Rh negative cells. The guinea pig experiments were inconclusive largely because of a high mortality due to an unknown epizootic. When purified preparations of elinin became available, animal experiments were performed again as described below: *Rabbits*: Five-tenths of a ml was injected into the ear veins of each of 5 rabbits over a period of 5 weeks. Three injections were given the first 3 days of the first, third, and fifth weeks. Ten days after the last injection samples of blood were removed by cardiac puncture from each animal. *Guinea pigs*: Group 1—Six guinea pigs were injected intraperitoneally with 1 ml of a 1% elinin solution in 0.15 M NaCl at 48-hour intervals for a total of 4 injections. Four days after the last injection the animals were bled by cardiac puncture. Five ml were removed from each animal, and 2 of them died after the operation. Forty days after the last injection the four remaining animals were given 1 ml subcutaneously and 48 hours later were given 1 ml intraperitoneally. Twelve days later they were bled by cardiac puncture.

Group 2—Six guinea pigs were injected as in Group 1 except that a 17-day interval intervened between the fourth and fifth injection.

Group 3—Six guinea pigs were injected as in Group 2, using 1 ml of packed type O Rh_0 red cells instead of elinin. The cells were washed 4 times with 3 volumes of saline before injection. The red cells of a single donor were used and were removed by venous puncture the same day they were injected. The presence of Rh antibodies in the sera of the

sensitized animals was determined after removing the nonspecific agglutinins with Rh negative cells. The red cells used to absorb the sera were obtained from three Rh negative, MN donors. The red cells of the three donors were mixed and washed 4 times with 2 volumes of 0.15 M NaCl. The blood removed from the animals was allowed to clot in the refrigerator and the sera removed after centrifugation. The sera were frozen and kept in dry ice until tested. After the sera were thawed they were heated at 56°C for 30 minutes and tested within 24 hours. The sera were absorbed with equal volumes of packed red cells for 30 minutes at 37°C. The absorbed sera were tested with the Rh negative red cells used for absorption, red cells from a different Rh negative type O MN donor, Rh_1 type O red cells and Rh_0 type O red cells. The latter were those from the donor whose red cells were injected into the guinea pigs. Two absorptions were necessary before the sera from the rabbits and guinea pigs injected with elinin were completely absorbed; 4 absorptions were necessary with the sera from Group 3 guinea pigs. The absorbed sera from the rabbits and the guinea pigs of Groups 1 and 2 failed to agglutinate the Rh positive red cells; the absorbed sera from three of the guinea pigs of Group 3 agglutinated the Rh positive red cells and the absorbed sera of the others failed to agglutinate them. Aliquots of the unabsorbed sera from the guinea pigs of Group 3 were diluted 15 times with 0.15 M NaCl and absorbed and tested as above. Two absorptions were necessary to remove the agglutinins for Rh negative red cells. One of the sera gave slight agglutination with Rh positive red cells after absorption and the remainder were negative. This latter absorption procedure was repeated using a 30% albumin solution as the diluent instead of NaCl and no agglutination was obtained with any of the sera and Rh positive cells.

Humans: Elinin was injected into human volunteers who had been previously sensitized to the Rh factor. Five of the 6 subjects were women who had been sensitized during pregnancy and had been delivered of one or more infants with hemolytic disease of the newborn. Two of the subjects were pregnant at intervals during the time of the injections. The sixth subject was a male, age 30, who had been sensitized by multiple Group O transfusions received during treatment of a combat injury. All the subjects had only univalent, Rh_0 (anti-D) type of antibodies in the serum. In 3 of the subjects the titer of antibodies was sufficient (1-64 or more) to demonstrate inhibition in the test tube by the Rh activity of the elinin injected. In all instances an attempt was made to compare the effect of injections of elinin with subsequent injections of whole Rh positive cells. Red cells were injected in the form of whole blood with 2.5% citrate. The blood was transfused without delay from donor to recipient. In one instance 1 cc of blood was given intravenously after producing complete hemolysis of the cells by mixing with 5 volumes of distilled water.

Calculations of yield of elinin from red cells indicate that 1 ml of 1% elinin is roughly equal in Rh antigen content to 3 ml of packed red cells. Serum inhibition studies with elinin and whole cells indicate that this calculation is correct. The injection of elinin with Rh activity into human subjects with anti-Rh antibodies was approached with considerable caution. Preliminary observations with intradermal and subcutaneous injections produced no immediate or delayed local reaction suggesting hypersensitivity. Thereafter intravenous and intramuscular injections were given without the occurrence of reactions suggestive of anaphylaxis, although intravenous injections were abandoned because of pyrogenic type of reactions. Following injection of elinin or whole blood, samples of serum for antibody studies were drawn at 7 to 10 day intervals. Antibody titrations were done with freshly drawn Rh_1 (CDe/CDe) cells. The serum was stored in the frozen state and antibody titrations were repeated when all sera pertaining to a single observation had been collected.

Results of human injection. In no instance was the injection of elinin into sensitized human subjects followed by a significant rise in antibody titer. These observations, which suggest a lack of antigenicity, are significant

TABLE II.
The Failure of Elinin with Rh Activity to Produce a Rise in Antibody Titer in Individuals Who Showed a Response to Subsequent Injections of Red Blood Cells.

Subject	Date	Antibody titer	Injection
W.M.	4/14/48	1-64	
	4/21	1-4	
	4/30	1-16	
	5/ 5	1-16	1.2 ml 1.5% Elinin I.V.
	5/14	1-32	1.0 ml Blood I.V.
	5/20	1-2560	
	6/16	1-8	
	9/30	1-2	3 ml 1.5% Elinin I.M.
	10/ 8	1-2	1.0 ml Blood I.M.
	10/15	1-3200	
	12/ 9	1-2	4.0 ml 2.5% Elinin I.V.
	12/20	1-2	
	4/15/49	1-512	1 ml hemolyzed blood I.V.
	4/22	1-512	
K.S.	1/13/49	1-64	1 ml 1.5% Elinin S.C.
	1/20	1-64	1 ml 1.5% Elinin I.V.
	1/28	1-128	
	10/13	1-32	1 ml Blood I.M.
	10/21	1-100	1 ml Blood I.V.
	10/27	1-1600	1 ml Blood I.V.
	11/ 3	1-16000	

TABLE III.
Antibody Titers Following Intramuscular Injection of 8% Solution of Elinin.

Subject	Date	Antibody titer	Injection 8-10% elinin
V.G.	Previous titers	1-4 to 1-128	
	2/21/49	1-32	5 ml I.M.
	2/23		5 ml I.M.
	2/24	1-32	
	3/ 1	1-32	
	4/19	1-64	
	4/22	1-64	10 cc I.M.
	4/26	1-64	
	4/30	1-64	
W.M.	6/ 4/49	1-1024	
	6/ 6	1-1024	10 cc I.M.
	6/10	1-512	
	6/15	1-512	
K.S.	3/28/49	1-400000	8 cc I.M.
	3/29	1-640000	9 cc I.M.
	3/30	1-640000	
	4/ 1	1-640000	
	4/11	1-640000	
	(Pt. delivered 9/49 of Rh negative baby)		
F.M.	6/ 4/49	1-1024	
	6/ 6	1-1024	10 cc I.M.
	6/ 7	1-2048	
	6/10	1-512	

in only 2 of the five individuals, since only 2 showed a rise in antibody titer following subsequent injection of red cells as shown in Table II.

Four of the 5 subjects were given intramuscular injections of elinin in amounts comprising the entire lot that could be produced in the ultracentrifuge in one week. In no subject was there a significant drop in antibody titer as shown in Table III.

Discussion. Our evidence to date indicates that elinin from Rh positive cells, while capa-

ble of neutralizing antibodies *in vitro*, is not antigenic when injected into animals or into human subjects, known to be capable of producing Rh antibodies. The failure to produce a rise in antibody titer in 2 human subjects who later responded to injections of whole red cells is a more convincing demonstration of lack of antigenicity than the failure to produce Rh antibodies in animals. It is interesting in this connection that one subject (W.M.) who showed a response to the injection of whole Rh positive cells failed to show a rise in antibody titer after the injection of freshly hemolyzed cells. Further observations are necessary to determine if the apparent loss of Rh antigenicity following hemolysis is partial or complete. The failure of the human subjects to show a drop in antibody titer following injections of elinin from Rh positive cells is not surprising. Calculations from serum inhibition studies indicate that 3 to 5 g of elinin will be needed to neutralize the circulating antibodies in an individual with an antibody titer of 1 to 64 if the relationship is stoechiometric *in vivo*. As yet the greatest amount given by I.M. injection is 1 to 1.5 g. In addition to the lack of quantity given we have no information as to the rate of absorption of elinin from an intramuscular depot or its antibody neutralizing activity when absorbed after exposure to tissue enzymes. The possibility that 3 to 5 g of elinin given intravenously would neutralize the circulating antibodies has not as yet been explored largely because of the difficulty of obtaining this amount of material and free from pyrogens. As stated above, we have not been able to obtain an active fraction of crude preparations of elinin by chemical or enzymatic means. This has also been true of the purified preparations of elinin. We have not been able to repeat the work of Carter(5), in extracting an active substance from whole red cells by alcohol and ether. The preparations obtained by her method of extraction yielded uniformly negative results with our method of assay. We believe that the basic constitution of the Rh antigen is as yet unknown. Observations of the complete inactivation of the Rh factor by trypsin suggest that the Rh hapten is very closely associated with a protein if it is not a protein itself.

Summary. 1. Elinin, a lipoprotein fraction of the red cell exhibiting Rh activity has been isolated in relatively pure form largely by physical methods.

2. Injection of elinin from Rh positive cells into animals has so far failed to yield a measurable titer of anti-Rh antibodies.

3. Injection of elinin into human subjects who have been sensitized to the Rh factor by pregnancy or transfusion failed to increase the titer of Rh antibodies in the serum. Two of the subjects injected showed subsequent response to injection of whole Rh positive cells.

4. Injection of 1 to 1.5 g of active elinin intramuscularly failed to effect a change in titer of circulating antibodies.

5. Carter, B. A., *J. Immunology*, 1949, v61, 79.

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Carbonic Anhydrase in Congenital Heart Disease.* (17772)

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Children with cyanotic congenital heart disease frequently tolerate relatively low degrees of oxygen saturation of the blood without any noticeable effects on the intelligence or on the state of consciousness when at rest. In connection with a study of the adaptive mechanisms involved, an investigation has been made of the possible role of carbonic anhydrase in the blood of such children. Car-

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