utilized: In the first series, a donor dog was included in the extracorporeal circulation to supply blood to the constant flow perfused, decapitate head; in the second series, the donor dog was replaced with an oxygenator-reservoir system. Results indicate that the direct effect of increasing $paCO_2$ and decreasing pH is a fall of cerebral vascular resistance and vice versa.

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Comparative Immunology: Natural Hemolytic System of the Spiny Lobster, *Panulirus argus** (33547)

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The phylogenetic development of immunity has come under increased study in recent years. The attention of our laboratory and others (1–8) was directed toward the study of immune mechanisms in animals at various levels of the phylogenetic tree. Recent work involved a number of invertebrate species as well as the lower vertebrates. It is hoped by such studies to gain insight into the evolution of various factors in mammalian immunity such as complement systems and the immunoglobulins.

Our present work deals with a marine invertebrate, the West Indian spiny lobster *Panulirus argus,* which possesses a natural hemolytic system for sheep erythrocytes, a natural hemagglutinin for various species of red blood cells, and an inducible bactericidin for gram-negative enteric bacteria (9). The present paper is a preliminary report of studies designed to characterize the naturally occurring hemolysin of the lobster in order to further define its biochemical and evolutionary significance.

Materials and Methods. Specimens of the West Indian spiny lobster, *Panulirus argus,* were collected in the waters surrounding Bimini, Bahamas and maintained in hardware cloth cages in 26–28° tidal sea water at the Lerner Marine Laboratory. The animals, weighing 300–500 g, were bled from the pericardial sinus with an 18-gauge needle inserted between the cephalothorax and abdomen. Lobsters bled in this manner yielded 15–30 ml of hemolymph which was collected directly into saturated sodium citrate to prevent

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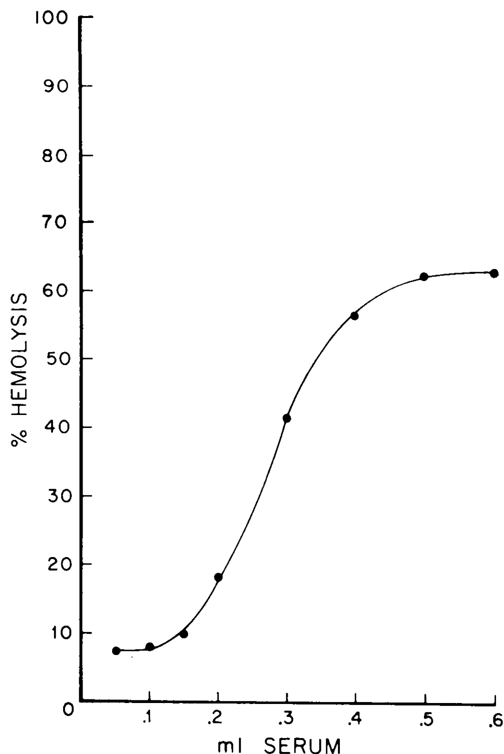

FIG. 1. Dose response curve: percentage of hemolysis of sheep erythrocytes plotted as a function of the volume of lobster hemolymph.

clotting (1 vol of saturated citrate/10 vol of hemolymph), and centrifuged within 30 min to remove the cells. Hemolymph to be used immediately was maintained in melting ice baths. Other samples collected for use at a later date, were frozen and stored at—25° to minimize loss of activity. Hemolymph kept in this manner for a 3-month period retained full activity.

The natural hemolytic activity of individual animals was evaluated by combining serum dilutions with a constant amount of sheep erythrocytes standardized spectrophotometrically as outlined in Kabat and Mayer (10). Each tube contained a dilution of serum, 0.25 ml of sheep erythrocyte suspension containing 1.0×10^8 erythrocytes/ml, and buffer to give a final volume of 3.75 ml. A standard isotonic NaCl–Veronal buffer, pH 7.3, of ionic strength 0.147, containing 0.00015 *M* Ca^{2+}, 0.001 *M* Mg^{2+}, and 0.1% gelatin was used for all dilutions. The degree of hemolysis was determined by using a Beckman-DB spectrophotometer at a wavelength of 413 mμ. At the conclusion of each assay the reaction tubes were centrifuged to remove unlysed cells and the percentage hemolysis was calculated by reading the OD of free hemoglobin and correcting for hemolymph color and cell blank. Both positive and negative controls were carried out for each assay. The positive control consisted of 0.25 ml of sheep erythrocytes in 3.5 ml of distilled water which provided a 100% hemolysis reference point. The negative control, used to detect any indiscriminate cell lysis, consisted of a similar amount of red cells added to 3.5 ml of standard isotonic buffer. All tubes were incubated at 37° for 90 min unless otherwise noted.

Results. The West Indian spiny lobster, *Panulirus argus,* possesses a natural hemolysin which showed a certain degree of specificity for sheep erythrocytes and a natural hemagglutinin for various species of red blood cells. The hemolytic titer varied according to the concentration of sheep red blood cells used as an indicator. Results from controlled assays using various erythrocyte concentrations revealed that 1.0×10^8 erythrocytes/ml gave optimal sensitivity in this hemolytic system.

Figure 1 illustrates a typical dose response curve that results from the addition of increasing amounts of lobster hemolymph to a constant amount of sheep erythrocytes. The range of the curve extends from 7% lysis at a final dilution of 1:75 to 62% lysis at a 1:6 dilution. Because of the high absorbancy due to hemocyanin in the hemolymph a relatively large correction factor for the two points nearest to the origin of this curve is necessary. As a result, these points are subject to a greater degree of experimental error, and therefore precise interpretation is not possible. From the sigmoid shape of the curve it is evident that 100% hemolysis is approached only gradually. In such a system, large increases in the amount of hemolymph would be required for total lysis. The 50% end point in this hemolytic titration is reached at a final dilution of 1:14 of the lobster hemo-

TABLE I. Lysis of Sheep Erythrocytes by Normal *P. argus* Hemolymph[a] at Various Temperatures after Incubation (18 hr).

Temp (°)	Hemolysis (%)
0	0
4	0
25	100.0
37	100.0

[a] One-half ml of a 1:7.5 dilution of hemolymph was used.

lymph. Attempts to increase the degree of lysis, and thus increase the sensitivity of the assay system, by addition of rabbit hemolysin were not successful. The use of various other species of antibody for this purpose is now under investigation. Although there is a natural hemagglutinin present in the lobster, whether or not it is necessary for the action of the hemolysin is as yet undetermined.

As indicated in Table I, the activity of this naturally occurring lobster hemolysin is temperature-dependent. Incubation of the hemolymph with a constant amount of sheep erythrocytes at 0 and 4° for an 18-hr period resulted in no detectable hemolysis. Incubation of a similar mixture at both room temperature and 37° for the same period of time gave 100% lysis. This inhibition of lytic activity at lower temperatures suggests the involvement of an enzymatic type reaction in this hemolytic system. If the mixture of sheep erythrocytes and hemolymph that exhibited no hemolysis at 0° is kept at this temperature, washed three times with cold isotonic buffer to remove the hemolymph, and then incubated at 37° for 90 min in a standard assay system, 100% lysis occurs suggesting that the hemolysin has been absorbed onto the red cell surface. Heating of the lobster hemolymph to 52° for 20 min resulted in complete loss of lytic activity. Other inactivation procedures involved the use of EDTA which also successfully inhibited the hemolysin. The hemolytic system remains after dialysis for 7 days provided that Ca^{2+} and Mg^{2+} are replenished. Absorption procedures using packed human and sheep cell stroma resulted in loss of hemolytic activity suggesting that the hemolysin is absorbed from the hemolymph by these techniques. Such procedures remove hemolytic activity even when carried out at 0° suggesting that the hemolysin can attach to the red cell membrane but, as shown by the next experiment, no lysis occurs at this temperature in a standard assay system.

The characteristic kinetics of this natural hemolytic system are illustrated in Fig. 2. A modification of the method described in Kabat and Mayer (11) was used for these experiments. Incubating samples were removed at various time intervals and placed immediately into a 0° water bath to halt the reaction. The samples were then centrifuged and the degree of lysis was determined. As shown by the resulting curve, hemolysis begins at zero time and does not reach an end point even after a 3-hr period of incubation. Thus it seems that in the lobster hemolytic system the time of incubation is an arbitrary factor.

Discussion. The data presented in this paper represent a preliminary report on a naturally-occurring hemolysin present in the hemolymph of the spiny lobster. The initial experiments reported here were designed to increase the sensitivity of the assay system in hopes of boosting the titer so that smaller quantities of the lysin could be detected. Manipulations involving red cell type, concentration, and quantity were carried out in an attempt to achieve optimal indicator conditions in this hemolytic system. A sheep red blood cell concentration of 1.0×10^8 erythrocytes/ml gave the greatest percentage of lysis in the assay used. Attempts were also made to increase titer by varying the ionic

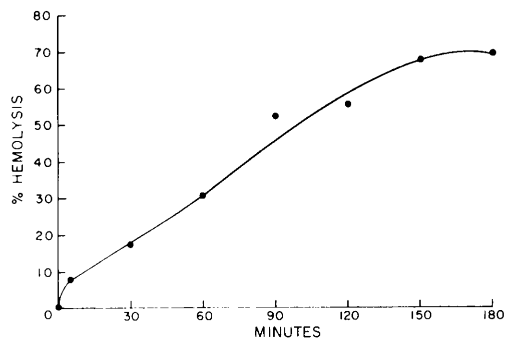

FIG. 2. Kinetics of hemolysis of sheep erythrocytes by lobster hemolysin at 37°.

strength of the buffer, but were not successful. In each titration the final reaction volume was 3.75 ml.

The sigmoidal dose response curve of the reaction between sheep erythrocytes and increasing amounts of lobster hemolymph indicates that a multiple step mechanism may be involved in this hemolytic system. Recalling the inability of the lysin to hemolyze cells at 0°, it is possible that the lytic system consists of a single protein species that is first absorbed onto the surface of the red cell, and then proceeds, by one or more subsequent steps, to lyse the cell. If the dose response curve of the spiny lobster is compared to similar data using sensitized sheep erythrocytes and guinea pig complement, definite similarities are seen to exist suggesting that the multiple step phenomenon characteristic of the guinea pig system is also common to the lobster hemolysin.

As shown in Fig. 2, the results of the kinetics experiment are also described by a sigmoid curve. In this plot of the kinetics of hemolysis of sheep erythrocytes by lobster hemolysin, it is interesting to note that an end point is not reached even after several hours of incubation. A similar situation occurs when human complement and sensitized sheep erythrocytes are reacted together. Such results indicate that the time of incubation is an arbitrary factor. These findings are in contrast to the guinea pig complement system in which a leveling off of hemolysis is observed after a 50-min period of incubation in a standard assay system. Again unlike the guinea pig system whose kinetics exhibit an initial lag period due in part to the need for activation of the first component of this system (C1), the lobster lysin shows no such lag, suggesting a simpler system requiring no activation step.

Perhaps one of the most interesting facts about the lobster hemolytic system is its inability to act at the lower temperatures of 0 and 4° over extended periods of time. In this regard, the lysin of the spiny lobster differs from the lamprey lysin reported by Gewurz *et al.* (12), which exhibits increased activity at lower temperatures. As noted previously, incubation of lobster hemolymph and sheep erythrocytes under conditions of lowered temperatures for an 18-hr interval resulted in no detectable lysis. Results such as these suggest that the actual mechanism of cell lysis may proceed by means of an enzymatic type reaction, possibly similar to one or more of the reaction steps found in the classical hemolytic complement system.

We are presently involved in the complete physical–chemical characterization of this lobster hemolysin in an attempt to determine if the hemolytic system of the spiny lobster is in any way related to, or precursory of, the classical mammalian complement system.

Summary. A preliminary investigation of a naturally occurring hemolytic system in the West Indian spiny lobster, ***Panulirus argus,*** showed the hemolysin to have a certain degree of specificity for sheep erythrocytes and a natural hemagglutinin for various species of red blood cells. The resulting sigmoid shaped dose response curve of the reaction between sheep cells and increasing amounts of lobster hemolymph suggests that a multiple step mechanism may be involved as is common to mammalian hemolytic systems. Results of kinetics experiments involving the lysin are presented and apparently differ from those characteristic of the classical guinea pig complement system. The lobster hemolytic system was heat labile, inactivated by EDTA, and temperature-dependent; the latter indicating the possible existance of an enzymatic-type reaction. Attempts to potentiate lytic activity by addition of rabbit hemolysin were not successful. Results suggest that further work will be required to determine if the lobster hemolysin is in any way analogous to one or more components of the classical hemolytic complement system, either in makeup or mode of action. Additional studies should also focus on the hemagglutinin to elucidate the role it plays, if any, in this hemolytic system.

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The Amount of Fetal Blood Remaining in the Placenta at Birth* (33548)

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For physiologic and medical reasons it is desirable to know the amount of fetal blood which remains in the placenta and umbilical cord when they are severed from the fetus at birth. This should be an approximation of the partition of blood between fetus and placenta during undisturbed intrauterine life. Data based on weight gain of the neonate by placental transfusion as reviewed by Smith (1) and Moss and Monset-Couchard (2) agree fairly well on an amount of approximately 100 g in a full-size infant. Estimates of the amount of blood remaining in the placenta, based on the volume drained through the cord, vary from 85 to 125 ml after early clamping of the cord, and from 0 to 40 ml after late clamping (2). Smith (1) has cautioned that such amounts are not necessarily representative of the content at any moment before the onset of labor.

Since none of the reported estimates of the fetal blood content of the placenta include the amount which cannot be drained through the cord, it is desirable to obtain a direct measure of the amount of blood separated from the fetus at birth. Whole placentas were homogenized and their blood contents determined. Since the placenta also contains maternal blood, determinations were limited to fetal hemoglobin and related to the concentration in cord blood from the same specimen.

Method. After the cord was cut at delivery between clamps, the clamp on the placental side was left in place, and the entire specimen was received in a pan in which the following dissection was carried out. A sample of unclotted cord blood was diluted with 4 times its volume of water, the resulting hemolysate was centrifuged and the supernatant examined for fetal hemoglobin by the alkali denaturation method of Singer *et al.* (3). The placenta specimen including cord and blood in the pan was treated as follows. The cord was stripped until all its blood had emptied into the pan, and was discarded. The same was done with the fetal membranes beyond the margin of the placenta. In order to reduce the amount of connective tissue for subsequent homogenizing in a blendor, the chorionic plate was dissected from the placenta, (care was taken to separate all red placental tissue and to empty the large fetal

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