STUDIES ON THE PROTEINS OF THE PLASMA PORTION OF THE BLOOD OF THE OMMASTREPHID SQUID, ILLEX ILLECEBROSUS ILLECEBROSUS (LESUEUR, 1821)

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STUDIES ON THE PROTEINS OF THE PLASMA PORTION OF THE BLOOD OF THE OMMASTREPHID SQUID, *ILLEX ILLECEBROSUS ILLECEBROSUS* (LESUEUR, 1821)

by

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"I finally saw the blood..."

-- Wm. Harvey

ABSTRACT

Specimens of the Ommastrephid squid *Illex illecebrosus illecebrosus* LeSueur were captured at two locations on the Avalon Peninsula of the island of Newfoundland, from July to October, 1966. A blood sample was obtained from each of the more than 400 specimens immediately upon capture. The blood samples were each centrifuged in the laboratory to remove cellular materials, and the plasma samples thereby obtained were stored at -20°C. Samples of this plasma were examined refractometrically for total protein content and subjected to electrophoretic analyses on a cellulose acetate medium. Antiserum to squid plasma was raised in a rabbit, and immuno-diffusion tests were carried out, also on cellulose acetate. The total protein values obtained ranged from 11.30 to 14.50 g/100 ml of plasma, the average being 12.76 g/100 ml. The electrophoretic analyses revealed two closely associated protein components, at least one of which is believed to be the proteinaceous part of the respiratory pigment, hemocyanin. The immuno-diffusion tests indicated the presence in the blood plasma of the squid of at least one antigenic component, probably hemocyanin.

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INTRODUCTION

Personnel of the Department of Biology are at present conducting an extensive research programme with respect to the biology of the squid *Illex illecebrosus illecebrosus* (LeSueur), commonly known as the "short-finned squid". As part of this programme, it was considered important to include a series of studies pertaining to the blood of this animal. The first such study, which forms the basis for this thesis, deals with the proteins of the plasma portion of the blood.

It is believed that *I. i. illecebrosus* is one of three subspecies of a genus of the teuthoid cephalopod Family Ommastrephidae (Phylum Mollusca), the other two being *I. i. coindetii* (Verany) and *I. i. argentinus* (de Castellanos). The latter was originally identified as *Ommastrephes argentinus* (de Castellanos, 1960) but was later placed in the species *I. illecebrosus* upon recommendation by G. L. Voss (de Castellanos, 1964).

The form here under consideration is an oceanic squid, that is, it spends the greater part of its life in that region of the ocean beyond the edge of the continental shelf (Clarke, 1966). All oceanic squids are placed by some authors in the Superfamily Oegopsida, distinguished by the absence of a cornea in the eye. Within this superfamily, several species of the Family Ommastrephidae migrate annually into inshore waters. Among these are *I. i. illecebrosus* and *I. i. coindetii*, both of which appear to move into shallower waters in search of food during adolescence (Akimushkin, 1963; Clarke, 1966).

Illex i. illecebrosus, hereinafter referred to as *Illex*, has been reported as far south as the Gulf of Mexico (Voss, 1956) and as far north as Hebron, Labrador (Squires, 1959). It is most frequently encountered in the waters around Newfoundland, and then usually from early July until late October. This squid is very important commercially as bait in the cod-fishery and is taken wherever available, mostly from the heads of the various bays around the east and south coasts of the island.

Very little is known regarding the life history of *Illex*. Reproductive activity has not been studied, primarily because the locations of the breeding grounds are unknown. Knowledge of reproduction in squid is based largely upon studies of the common Atlantic coast squid *Loligo pealei* LeSueur. The process is probably similar for *Illex*, though the type of location chosen for breeding purposes may differ, due to the oceanic habits of *Illex*.

The functional axes of the bodies of the squids and the other cephalopods do not correspond to the morphological axes, the latter being the same as those of the other members of the Phylum Mollusca. In the evolution of the Cephalopoda, the body became lengthened along the morphological dorso-ventral axis; and, as a result of a change in the manner of locomotion, this axis became the functional anterior-posterior axis (Barnes, 1963). The head with its circle of appendages is thus located at the functional anterior end of the body, and the fins at the functional posterior end. Hereinafter, reference will be made to the functional axes and aspects only when discussing pertinent morphological features of *Illex*.

Eight of these appendages are of equal and fixed length and are called arms; the remaining two are considerably longer and extensible, and are usually referred to as tentacles or tentacular arms. Each tentacle possesses suckers only on its clubbed, flattened terminal portion, whereas the eight arms bear suckers along their entire length. At the tapered posterior end of the body are located the triangular fins, whose apices are directed posteriorly and whose bases join the body at a point about one-third of the body length as measured from the posterior end. On the ventral surface the hyponome or funnel extends anteriorly from beneath the anterior edge of the mantle; it is involved in the propulsion of the squid and in other functions requiring the evacuation of water from the mantle or pallial cavity. A pair of eyes is located on the lateral surface of the head. All of these salient basic features of the external anatomy may be seen in Figure 1.

To better understand the significance of the distribution of the circulatory medium in *Illex*, it is considered necessary to present a brief resume of features of the internal anatomy of this squid. The internal organs of *Illex* may be shown to best advantage by slitting the mantle along the mid-ventral line, thereby exposing the mantle cavity (Fig. 2).

The most conspicuous organ thus revealed is the digestive gland, commonly called the "liver". Shaped roughly like a narrow cone, it has its base at the level of the mantle collar and extends posteriorly for about one-half the length of the mantle. Lying along the ventral surface of the digestive gland is the ink sac, over which runs the intestine. The

Fig. 1. *Illex illecebrosus illecebrosus*, external views:
A. ventral B. dorsal

a. Arm
b. Sucker
c. Head
d. Hyponome
e. Mantle, ventral surface
f. Fin
g. Tentacle
h. Eye
i. Tentacular club
j. Mantle, dorsal surface

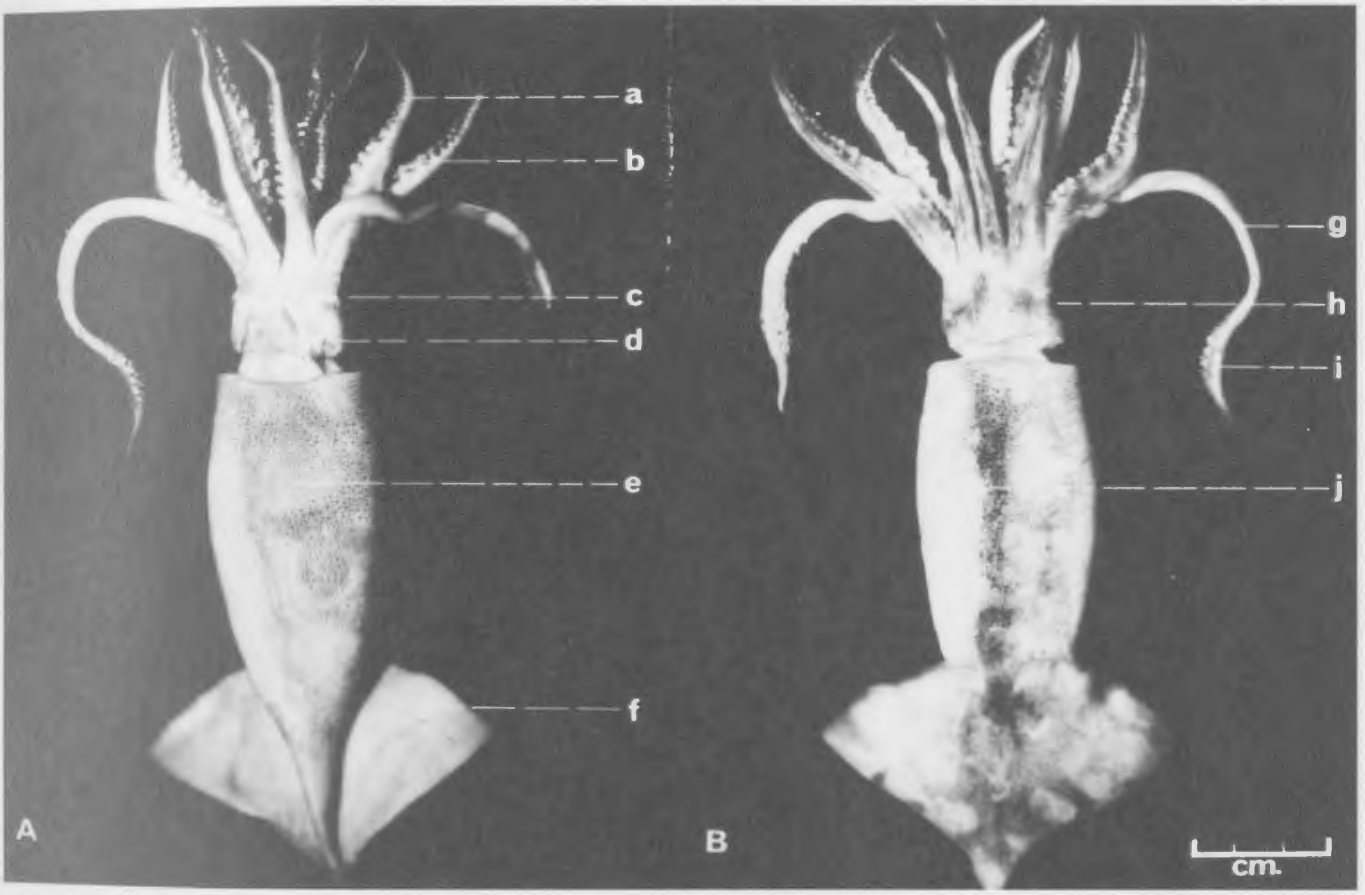
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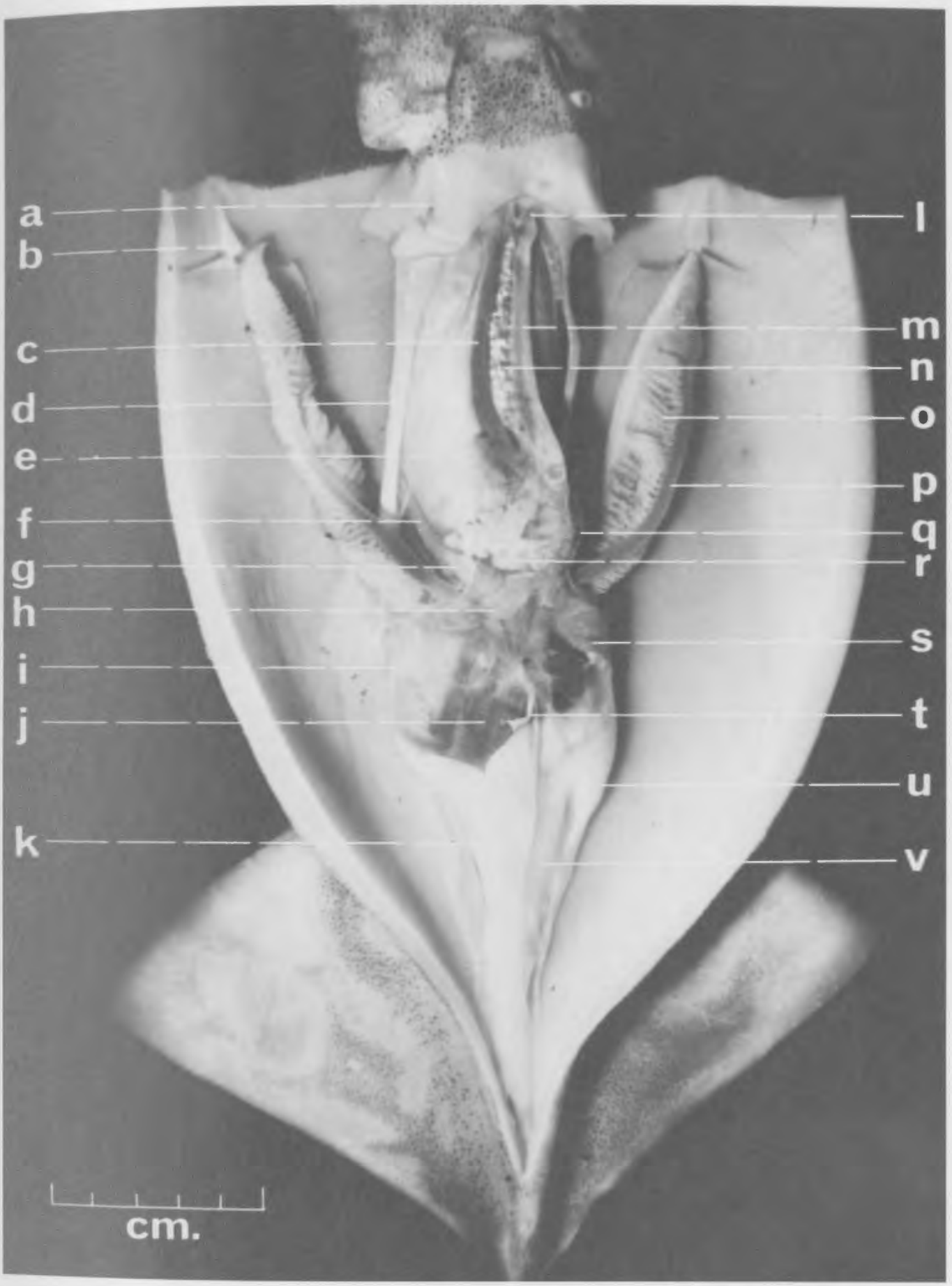
Fig. 2. *Illex illecebrosus illecebrosus*, male, internal view.

a. Mantle-locking socket
b. Mantle-locking stud
c. Esophagus
d. Retractor muscle
e. Hepatic portion of digestive gland
f. Cephalic artery
g. Systemic heart
h. Nephridium
i. Caecum
j. Stomach
k. Testis (spermary)
l. Anus
m. Rectum
n. Ink reservoir
o. Ctenidium
p. Efferent branchial vessel
q. Nephridiopore
r. Pancreatic portion of digestive gland
s. Branchial heart
t. Medial mantle artery, proximal portion (cut)
u. Spermatophoric sac
v. Posterior vena cava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latter extends for a short distance into the funnel and terminates at the anus, which is flanked by two anal papillae. The duct from the ink sac joins the rectum near the anus.

The esophagus, due to its position, is not visible in Fig. 2. After emerging from the buccal mass in the head, this narrow tube passes through the anterior portion of the digestive gland and then extends, in a position dorsal to that gland, posteriorly to enter the stomach. The stomach and the caecum together occupy a major part of the posterior region of the mantle cavity. The caecum is a larger, thin-walled saccular organ; the stomach lies to the right of the caecum, sometimes overlapping it slightly, and has muscular walls. The blind end of the caecum extends toward the posterior end of the mantle cavity; from the opposite end of the caecum the rectum extends anteriorly over the ventral surface of the digestive gland and ink sac, as already seen.

There are two ctenidia or gills present, one on each side of the mantle cavity; they are attached by mesenteries to the dorsal mantle wall and have their apices extending anteriorly. At the base of each gill is a small branchial heart. The systemic heart is located over the right ventro-lateral surface of the digestive gland, near the base of the right gill. Most of the blood vessels are not visible in Fig. 2, due to their transparency. It is possible, however, to see the medial mantle artery and the large postcaval veins; the importance of the latter to this research is discussed under Materials and Methods. The circulatory system as a whole will be considered later here.

The pancreatic portion of the digestive gland is a bilobed, diffuse organ lying over the ventral surface of the hepatic portion of the digestive gland in the area between the bases of the ctenidia. The nephridium or "kidney", is located in the mid-line over the ventral surface of the "liver" or hepatic portion of the digestive gland, between the two lobes of the "pancreas". The rectum extends between the nephridium and the digestive gland.

In the female, the ovary occupies the posterior apex of the mantle cavity, as does the testis in the male. There are two oviducts; each runs anteriorly and laterally from the ovary to a point just posterior to the branchial heart; here it becomes enlarged to form an oviducal gland and then terminates at a flared oviducal opening. Lying in the mid-line over the ventral surface of the stomach and caecum in the female are the paired nidamental glands which serve, at the appropriate time, to form a protective, jelly-like matrix to enclose the eggs.

In the male, the single testis or spermary occupies the position corresponding to that of the ovary in the female, that is, the posterior end of the mantle cavity. The coiled spermatophoric gland lies to the left of the anterior part of the caecum and extending posteriorly from the gland is the spermatophoric sac. The penis runs anteriorly from the anterior end of the spermatophoric sac and, after passing along the space between the rectum and the gill, terminates near the anus. Just to the left of the testis is the sperm bulb, which is the enlarged portion of the vas deferns; the latter is a convoluted tube which conducts sperm to the spermatophoric gland.

The circulatory system of *Illex* is very similar in general pattern to that of the common squid, *Loligo pealei* LeSueur, the latter having been described by Williams (1902). Mollusca are generally characterized by an open circulatory system. In such a system the movement of the blood is at least partially dependent upon a heart which receives blood from venous sinuses and pumps it out again through arteries, from which the blood percolates through the tissues back to the venous sinuses. However, the Cephalopoda are exceptional in this regard, in that they possess a closed circulatory system with capillaries interposed between arteries and veins. Furthermore, a separate branchial circulation exists between the collecting vessels and the systemic heart.

In the squid, a single branchial heart is located, as already mentioned, a short distance posterior to the base of each ctenidium (Fig. 2). These branchial hearts receive deoxygenated blood from the tissues via the paired anterior and posterior venae cavae. This blood is then pumped into the ctenidia, where the oxygenation process is reflected in a marked change in the colour of the respiratory pigment, hemocyanin, from bluish white to pastel blue, a process readily observed by opening the mantle of the living animal. A single branchial vein conveys the oxygenated blood from each ctenidium to the systemic heart located near the mid-line, between the bases of the ctenidia. The systemic heart pumps the blood, via the cephalic artery and the posterior aorta and their branches, to the many capillaries which make intimate contact with the tissues. These capillaries return the deoxygenated blood to the numerous veins which are really tributaries of the

venae cavae. The circuit of the blood is thus completed within a completely closed vascular system (Williams, 1902).

The bloods or corresponding fluids of all vertebrates and higher invertebrates are basically of the same general composition, consisting of numerous formed elements suspended in an aqueous medium known as plasma. Dissolved in this plasma are various electrolytes, non-electrolytes (such as lipids, glucose and metabolic wastes), proteins, and organic acids. In most animals, some of the protein of the plasma is in a conjugated form, as in certain complex substances which have the ability to combine with oxygen and transport it to the tissues of the animal. Since these substances impart a characteristic colour to the fluid, they are known as respiratory pigments. In certain groups, the respiratory pigments are dissolved in the plasma, while in others they are found only within cells suspended in the plasma. In the latter case, the plasma itself is usually comparatively colourless.

All molluscs except the members of the Class Cephalopoda have open circulatory systems, in which capillaries are lacking and the circulating fluid has intimate contact with the interstitial tissue fluid; it is probable that considerable mixing of these two fluids occurs regularly. The term "hemolymph" has been used to designate the circulating fluid of such animals. However, in the case of the cephalopods, in which the vascular system is a closed circuit (or circuits), the term "blood" is more appropriate and has been used by many authors (e.g. Nicol, 1967). The hemolymph or blood of molluscs and of other invertebrates has been the subject of considerable

research, some of which will now be reviewed very briefly.

Robertson (1949) carried out an extensive study of the inorganic constituents of the "blood" of numerous invertebrates. An analysis of the circulating body fluid of 20 species belonging to five different phyla revealed evidence of some ionic regulation within each species. However, the most highly developed regulatory ability was found in certain species of decapod Crustacea and of cephalopod Mollusca. The lower forms were found to have, in their hemolymph, ions at approximately the same concentration as in the sea water in which the animals live, while the decapods and cephalopods were able to maintain ionic concentrations significantly different from those in the environment. In the cases of the octopod *Eledone cirrosa* (Lamarck) and the cuttlefish *Sepia officinalis* L., Robertson concluded that differential excretion by highly developed renal organs is an important factor in ionic regulation. He found that Na^+ and SO_4^{--} ions are eliminated in greater quantities than those which would be present in a simple ultrafiltrate of the blood plasma, so that the concentrations of these ions in the plasma are lower than in sea water. Conversely, K^+, Ca^{++}, Mg^{++} and Cl^- ions are excreted in proportions below those of an ultrafiltrate, which elevates their concentrations in the blood.

The protein content of the blood of certain crustaceans and molluscs has been presented by Dittmer (1961), using several sources in the literature. While the values for the crustaceans and lower molluscs are generally low, those for the cephalopods are much higher:*

*However, Cohen (1968) reports a total protein value of 12.6 g/100 ml of hemolymph for the coconut crab, *Birgus latro*.

Homarus americanus	4.28 g/100 ml of plasma
Ostrea edulis	0.02 g/100 ml " "
Loligo forbesi	14.97 g/100 ml " "
Sepia officinalis	10.90 g/100 ml " "

In some species of crustaceans and insects, damage to a blood vessel results in the formation from proteins in the plasma, of a fibrin clot, in a manner similar to the hemostatic process found amongst vertebrates. In such cases, the fluid remaining after the formation of the clot may be called serum. In the majority of invertebrates, however, loss of body fluid from a wound is prevented solely by the formation of a cellular thrombus. Nicol (1967) reports that in molluscs, as in polychaetes and echinoderms, hemostasis is achieved by means of aggregation of amoebocytes at the wound. In these animals, the plasma does not clot (Meglitsch, 1967) and therefore the term "plasma" is, I believe, more accurate than the term "serum" in referring to molluscan blood from which the formed elements have been removed.

In *Octopus vulgaris* Cuvier, Lange (1920) found that if an arm was cut off or cast off, there was no bleeding. She presumed that, at the moment the arm was severed, the blood vessels contracted at the site of the wound. At the same time the skin around the rim was observed to curl inward, covering the circumference of the wound. Approximately six hours later, according to Lange, the muscles of the blood vessels relaxed and blood slowly entered the wound, the blood cells agglutinating to form a protective clot-like cell mass over the central area.

Another role of plasma protein in the blood of some invertebrates, according to Nicol, is that of ionic regulation. Cations may be bound by negatively charged protein molecules, which cannot diffuse across bounding membranes due to their large size.

The most important function of plasma proteins in molluscs is the formation of the respiratory pigment. Over 90% of the total protein may be included in this conjugated protein which, in addition to its respiratory functions, also serves as the most important buffer in maintaining the optimum hydrogen ion concentration of the blood (Prosser and Brown, 1961).

Most molluscs have hemocyanin as the respiratory pigment of their blood. The hemocyanins are actually a series of protein and copper complexes having high molecular weights, and are found only in solution in the plasma. In many cases, several molecular sizes are indicated, the larger molecules being aggregates of definite numbers of the smallest ones (Prosser and Brown, 1961). In *L. pealei*, molecular weights as high as 3,700,000 have been estimated (Ghiretti, 1966).

The copper in hemocyanin is probably bound directly to the protein. Neither heme nor any other prosthetic group is present. The presence of the copper enables the hemocyanin to combine reversibly with oxygen and to function as a respiratory pigment (Ghiretti, 1966). The oxygen combines in the ratio of one oxygen molecule to two copper atoms (1 O_2 = 2 Cu); it is probable that the oxygen molecule is held between the two copper atoms. In *L. pealei*, the oxygen-combining capacity of the blood is 4.2 volumes per cent. Approximately 92% of this oxygen is removed during the course of circulation.

The hemocyanin operates near capacity at all times. It is nearly colourless when deoxygenated and blue when oxygenated, the shade of blue deepening with increased oxygen saturation (Prosser and Brown, 1961).

The copper in deoxygenated hemocyanin is in the cuprous oxidation state. It is believed to undergo a reversible valence change upon the oxygenation of the hemocyanin (Manwell, 1960). The copper content of the plasma is greater in cephalopods than in the smaller, less active molluscs. Molluscan blood combines with 1 to 7 mg O_2/100 ml of plasma, compared with a range of 6 to 15 mg O_2/100 ml in the blood of vertebrates (Meglitsch, 1967).

Little is known of the formed elements, the blood cells, of molluscs. Cells are certainly present, but little progress has been made in the classification of cell types. Amoeboid lymphocytes and phagocytic granulocytes seem to be universal in molluscs, while some molluscs also possess neutrophils and eosinophils. Differentiation of blood cells probably occurs within the blood itself (Meglitsch, 1967). As already mentioned, the clumping of blood cells at injuries is the primary means of achieving hemostasis in molluscs.

The vascular system of any animal is of such vital importance that an understanding of the biology of that animal must include an understanding of its circulating body fluid and of its constituents. It would seem most logical to begin by studying the fluid medium itself in order to first prepare an environment, so to speak, for the study of the formed elements, which must naturally follow. As a preliminary step toward an understanding of the plasma of the blood of the squid *Illex*, it was decided to undertake a

series of three studies on the plasma proteins. The importance of proteins in the blood of invertebrates, and especially in that of the Mollusca, has already been discussed. These studies include:

1. Electrophoretic separation of the proteins.
2. Total protein determination.
3. Double diffusion studies on antigenic components in the plasma.

The analysis of the protein content of the *Illex* plasma by means of electrophoretic separation forms the major part of this research project. It seems in order at this point to discuss briefly the theory underlying the technique to be described in the following section.

A mixture of similar substances may be better understood if the components of the mixture are separated from each other. Several laboratory techniques for performing such separations fall under the heading of chromatography, so named because the first applications of this method involved mixtures of coloured compounds. One of these techniques involves the use of an electric field as the separating force and is hence called electrophoresis.

When a solution containing charged particles is subjected to an electric field, these particles migrate; the positively charged particles move toward the cathode and the negatively charged particles toward the anode. This is the basis of electrophoresis. Proteins were among the first compounds to be separated by this technique (Van Norman, 1963). This

separation is possible because proteins, like their constituent amino acids, can behave as either acids or bases, or as dipolar ions (zwitterions). In the last case, the protein, having equal numbers of positive and negative charges, is said to be at its isoelectric point and is electrically neutral. However, in a strongly acidic or strongly basic solution, the protein molecule has a net positive or negative charge, respectively, and therefore migrates toward the electrode of opposite charge.

The rate of migration of the protein molecule depends upon the magnitude of the net charge on the molecule, which in turn depends upon both the dissociation of acidic groups and the binding of buffer ions. Proteins having different isoelectric pH values will, in a buffered solution, possess different net electric charges due to different degrees of dissociation of their acidic groups. Therefore, if an electric current is passed through a complex mixture of proteins in a buffered solution, such as that found in blood plasma or serum, the proteins will migrate at different rates and will consequently be separated into a number of fractions. A fraction may consist of a single protein or of several proteins having similar electrophoretic mobilities (Dessauer and Fox, 1964). Electrophoretic analysis of a medium such as plasma, containing proteins in fairly constant quantities, results in a characteristic pattern which, besides indicating the number of protein fractions present, may also provide clues as to the nature of those proteins.

The determination of the total quantity of proteinaceous substances present in the blood plasma of a lower animal such as *Illex* probably has far less absolute value than the same sort of analysis carried

out on human blood serum in a clinical laboratory, where the technique is routine. For this reason, the second phase of this research is to be regarded as being of secondary importance, in that the results produced do not in themselves provide much information. The results do, however, add to the significance of those obtained by electrophoretic analysis, as the latter technique does not in itself deal in absolute quantities, but rather in relative values. Furthermore, the measurement of total protein of molluscs provides a means of comparison with other molluscs with regard to the relationship between level of activity and concentration of respiratory pigment since, as we have seen, nearly all of the protein is in this conjugated form. A brief discussion of basic principles will be included together with a description of the instrument used, in the following section.

The study of the antigenic components of plasma and serum has become of such importance in the field of systematic serology (Boyden, 1953) that it was considered worthwhile to include such a study in this research programme. Here, again, a discussion of pertinent aspects of basic theory is in order, based on modern information presented by Haurowitz, (1968).

An antibody is a proteinaceous substance produced in the body in response to a specific stimulus. This stimulus is provided by the invasion of the body by a foreign substance, such as a protein or a polysaccharide, which in such cases is called an antigen. Though there are some exceptions, antibodies in general are proteins of the γ -globulin type. Each antibody,

once formed, has a specific affinity for the homologous antigen, that is, the antigen which originally stimulated the production of the antibody.

A substance which is antigenic to one organism may have no effect whatsoever on another. Antibody formation thus depends on both the antigenicity of the introduced substance and the responsiveness of the organism being invaded.

The agent which triggers the production of an antibody is called an antigenic determinant. A macromolecular antigen can possess several determinant groups; each one of them gives rise to the formation of a different type of antibody. An antibody, even if formed in response to a single antigen, may be a mixture of antibody molecules of similar, but not identical, composition. The antibody combines with the hemologous antigen and thus eliminates the threat to the well-being of the invaded organism. The combining sites of antibody molecules are complementarily adapted to the antigenic determinants, so that the antibody molecule reacts only with definite chemical groups (the determinants) in the surface of the antigen and not with the entire antigenic macromolecule.

Most antibody production in vertebrates occurs in those organs of the body which contain cells of the reticuloendothelial system and the lymphoid system. Antigen which has been injected is found mostly within phagocytic cells. However, most antibodies are known to be produced in plasma cells, which are lymphoid cells and are not phagocytic upon antigens. It is therefore apparent that at least two cell types are involved in the process of antibody production: (1) phagocytic and (2) lymphoid.

Typical antibodies have been found only in vertebrates. The reason for this is unknown. It has been suggested that invertebrates may lack a particular type of lymphoid cell which has the property of producing antibodies.

In order to determine whether a certain substance contains antigenic components, it is necessary to demonstrate the presence of antibodies in the blood of the animal into which the substance has been injected. Since the chemical structure and properties of antibodies are those of the typical γ -globulins, they cannot be differentiated from the "normal" γ -globulins by means of chemical methods. Detection of an antibody in a solution can be accomplished only by using the homologous antigen as a specific reagent, in order to observe the result of the combination of the antibody with the antigen. The simplest test of this sort is the precipitin test, which depends upon the insolubility of most antigen-antibody complexes in neutral isotonic salt solutions.

Various workers have developed special techniques for carrying out the precipitin test reaction. Some of these are the interfacial test or "ring test", the single gel diffusion test of Oudin, and the double gel diffusion test of Oakley and Falthorpe, the details of which are given by Haurowitz (1968). One of the more recent variations on the basic technique is the two-dimensional gel diffusion test developed by Ouchterlony in 1958.

In the Ouchterlony method as described by Haurowitz (1968), liquefied agar gel is poured into petri dishes. Solutions of antigen and

antibody are placed into holes, or wells, of uniform size cut into the agar after it has gelled. Usually a central well containing antibody is surrounded by several other wells containing the antigen. The solutions are left to diffuse through the gel toward each other, for several days, during which time a precipitate should appear in the region between antigen and antibody, indicating the formation of an antigen-antibody complex.

Diffusion of a single antigen toward a single antibody results in the formation of a single line of precipitate. If a mixture of two or more antigens diffuses toward a serum containing two or more corresponding antibodies, two or more precipition lines are formed. When two or more wells on the periphery of the gel plate contain identical antigens, a single common continuous zone of precipitate is formed. If the antigens in the two adjacent wells are different, and if the central well contains a mixture of the two corresponding antibodies, two different precipitin zones are formed which cross each other without fusing. The position of the precipitin band in the region between the antigen and antibody depends upon the diffusion coeffecients, which in turn depend upon the molecular weights of the antigen and antibody.

It was hoped that the research programme as outlined above will serve a two-fold purpose. The first, as already stated, was that of providing some understanding of the plasma portion of the blood of *Illex*. The second was that of establishing a basis for possible future work, an area of the systematics of the genus *Illex*, which may in time clarify the matter of the number of subspecies within the genus.

MATERIALS AND METHODS

Capture of Specimens

Specimens of *Illex* for use in this project were taken at two locations, both on the Avalon Peninsula (Fig. 3). The first is the head of an inlet known as South Arm, Holyrood Bay, Conception Bay, near the community of Holyrood. The second is a cove in Freshwater Bay, south of the harbour of St. John's.

Several different types of boat were used for the operations. At Holyrood these included: fishermen's dories; a chartered 35-ft. cabin boat, the "Bluefin"; and the M.V. "Marilyn Marie", Captain M. P. Whelan in command, under charter to the Industrial Development Service of the Canada Department of Fisheries. The chartered boat used at Freshwater Bay was the M.V. "Edwina Mary", commanded by the late Captain James Crockwell.

The gear used to capture the squid is essentially the same as that used by the local fishermen and is of two types. In the traditional method, dating back several generations, a single jigger is used. This is a weighted metal device of local design, patented by Neyle-Soper Hardware Co. Ltd. (Fig. 4.B). Its method of fishing is described by Williamson (1965). The more modern method involves a mechanical jigging apparatus, built locally according to a Japanese design (Quigley, 1964) (Fig. 4.A). The latter was found to be most effective whenever the squid were plentiful.

Fig. 3. A. Island of Newfoundland and adjoining Canadian mainland.

B. Section of the Avalon Peninsula, showing the two areas where samples were collected.

a. Area shown in B
b. Province of Quebec
c. Province of New Brunswick
d. Gulf of St. Lawrence
e. Island of Newfoundland
f. Conception Bay
g. Holyrood
h. South Arm, Holyrood Bay
i. St. John's
j. Freshwater Bay

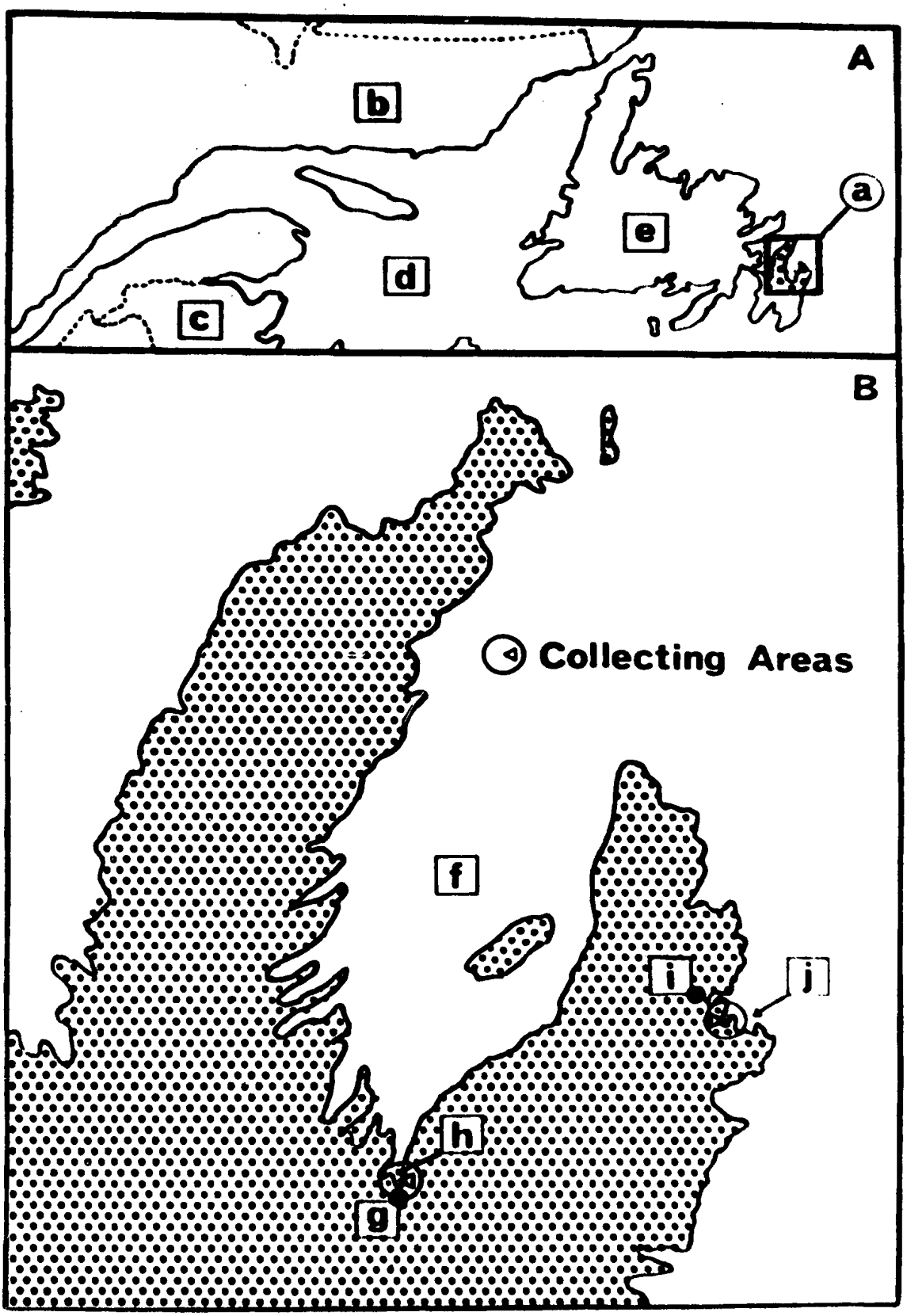
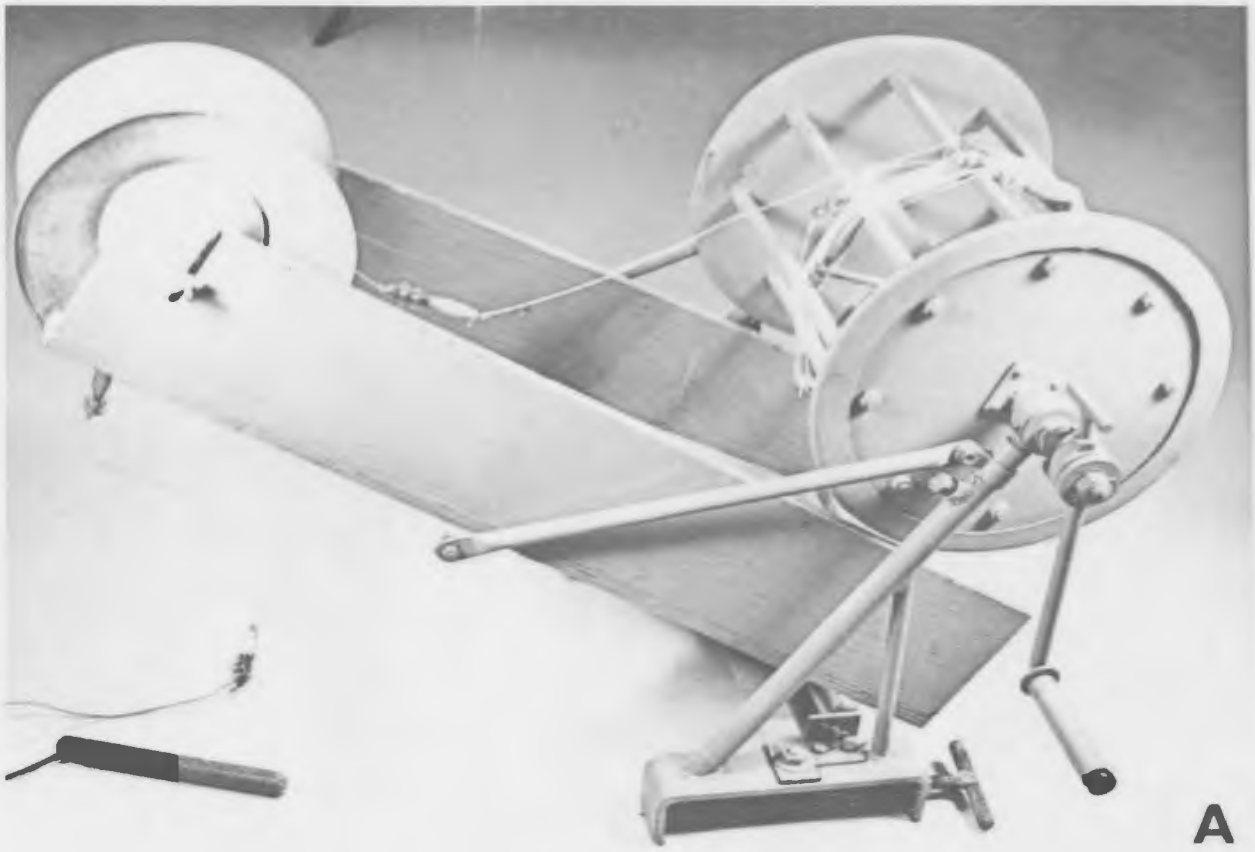

A
b
a
e
d
c
B
Collecting Areas
f
i
j
h
g

Fig. 4. A. Japanese-designed mechanical squid-jigger.

b. Weighted metal squid-jigger of local design, patented by the Neyle-Soper Hardware Co. Ltd., St. John's.



A

B

Obtaining Blood Samples

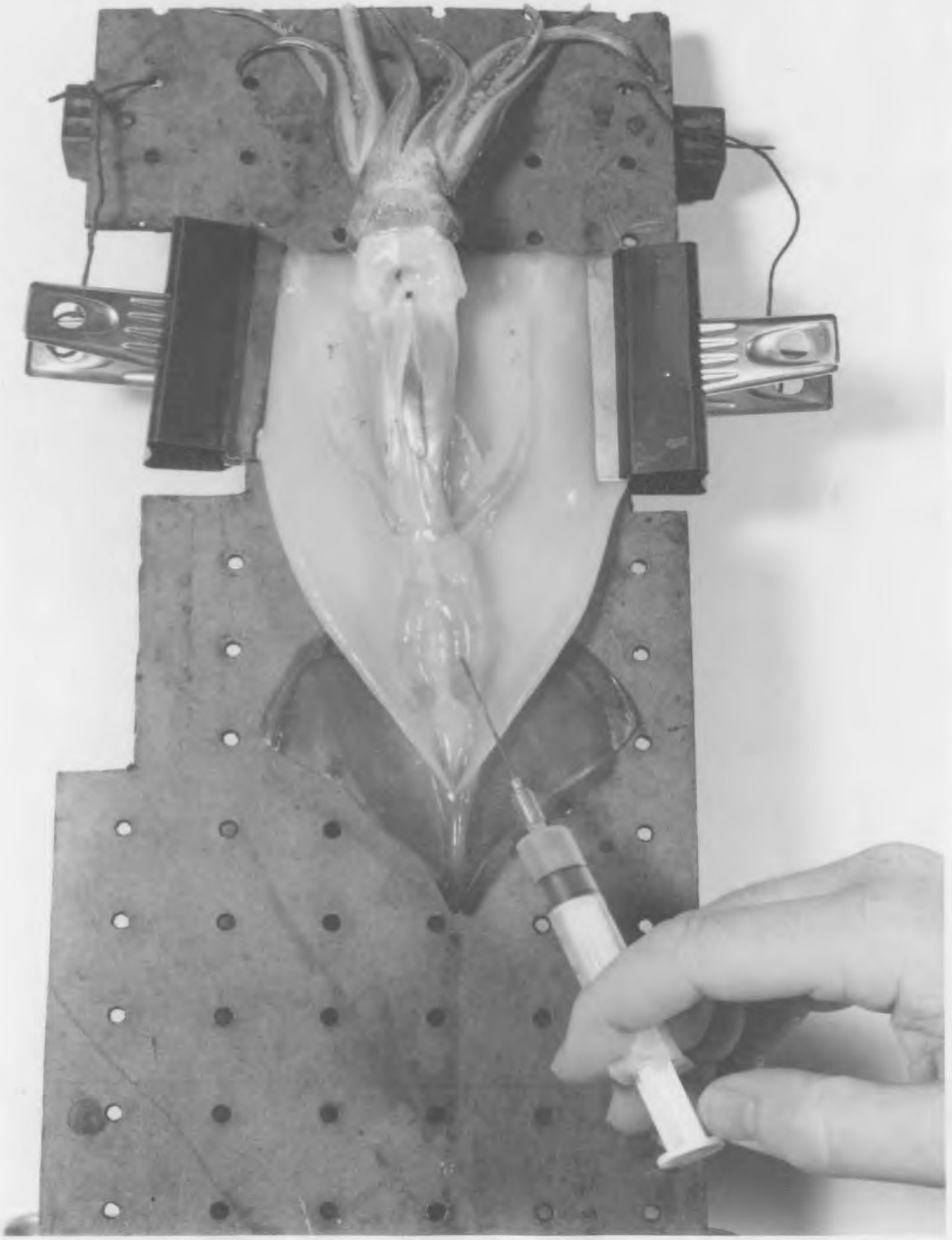
In order to obtain a sample of blood from an animal, it was first necessary to expose the internal organs. This was done by cutting open the mantle or pallium along the mid-ventral line, as for Fig. 2. The movements of the squid were restricted by placing it with its dorsal surface downward on an operating board (Fig. 5) and pinning back the cut edge of the mantle on each side. The design of the operating board was suggested by Mr. M. J. Mercer, a Scientific Assistant at the Marine Sciences Research Laboratory. It consists of a piece of light peg-board, cut as shown, and two spring-loaded paper clips, tied to the board to prevent loss. Because of its cut-out design, the board can accommodate squid of different sizes. The holes facilitate drainage.

The most convenient sources of blood were found to be the posterior venae cavae. By supporting the head end of the board to hold the animal in an oblique position, I could usually cause blood to pool in these vessels. The blood was withdrawn by inserting a 20-gauge, 1½ inch hypodermic needle attached to a 5- or 10-cc syringe into each vessel in turn.

The total volume of blood obtained from a single animal ranged from less than 1 cc to more than 6 cc, depending on several factors. The most important of these, as might be expected, was the size of the squid itself, the larger animals generally yielding the greater volumes. Also important, however, was the quantity of material present in the caecum. In the case of a greatly distended caecum, I always found it difficult or impossible to get the venae cavae to fill properly due, perhaps, to the pressure on

Fig. 5. Withdrawal of blood sample from left posterior vena cava of *Illex illecebrosus illecebrosus*.

Since it has been observed that *Illex* blood has no clotting mechanism (see page 77), precautions to negate clotting during withdrawal of blood are not necessary.



the walls of the vessels. I also found that a male squid nearly always yielded less blood than did a female of the same size. It has been observed (Bradbury,[1] personal communication) that the male testis occupies a greater volume than the female ovary, in squid of similar size. As in the case of the caecum, this may result in greater pressure on the walls of the venae cavae in the male and consequently in a smaller volume of blood present in these vessels. It should be explained that at no time did I attempt to completely exsanguinate a squid. Such a procedure, if possible at all, would certainly have required much time and a more sophisticated technique, which would have greatly reduced the number of samples obtained. I felt that it was more practical to collect as many different samples as possible, even though some blood remained in the hearts and smaller vessels of each animal following the removal of each sample from the posterior venae cavae. Therefore, the total sample from a given animal represents that amount of whole blood immediately collectable from the venae cavae and, to varying extent, closely allied blood vessels.

Determination of sex in *Illex* is relatively simple. In the male, the large whitish testis is visible at the posterior end of the mantle cavity; to the left and slightly anterior is the spermatophoric gland, though this may be less prominent in immature specimens (Fig. 2). The female is readily identifiable by the paired nidamental glands in the mid-ventral line, overlying the stomach and caecum; and by the paired oviducal openings, each of which is located a short distance posterior to one of the branchial hearts. Immediately after use each syringe was marked according to the sex of the animal from which the blood had been taken, and a new syringe was used for

[1]Miss Helen E. Bradbury, Memorial University of Newfoundland.

each subsequent sample.

The blood samples, still contained in their respective syringes, were quickly transported to the laboratory and stored temporarily, sometimes overnight, in a refrigerator at a temperature of about 5°C. As soon as possible each sample was centrifuged at 1750 rpm to remove the cellular material and any debris which might be present. The plasma was decanted carefully and each sample maintained, until used, at -20°C. Large samples were divided into two or three aliquots thereby making it unnecessary to thaw out the whole sample in order to obtain one or two millilitres for analytical purposes.

Experimental Methods

1. Electrophoresis

(a) Basic Technique

The basic technique of electrophoresis for analysis of proteins was first developed by the Swedish scientist Arne Tiselius. The original method, using a U-tube with an electrode at each end, has been modified many times to suit particular applications. One of the modern techniques is known as zone electrophoresis; it has the advantages of simplicity and the use of only small quantities of sample material. The protein-containing solution is applied to an inert porous material moistened with buffer; the latter transmits the electric current and maintains the correct pH. Following a period of time sufficient for adequate separation, the protein fractions are made visible by a protein-

specific stain. Examples of supporting media which have been used quite successfully are silica gel, starch gel and filter paper (Jellinck, 1963). Kohn (1967) described a new medium for zone electrophoresis: strips of pure cellulose acetate. This was the method used in this research.

(b) Buffer Solution

The buffer used in these analyses was a barbitone buffer described by Owen (1956), having a pH of 8.6. The formula is as follows:

Sodium diethyl barbitone ----------	5.0 g
Sodium acetate (hydrated) ----------	3.25g
Hydrochloric acid 0·1N ----------	34.2 ml
Distilled water, to ----------	1,000 ml

This buffer was made up in 1,000 ml quantities and kept for periods up to one month, during which the pH was not found to change appreciably. Quantities required for an electrophoretic run were removed from the stock bottle (kept at 0^{o} to $10^{o}C$) and afterward returned to it. The stock of buffer was divided into two portions: a quantity of 200 ml at full strength for impregnation of the cellulose acetate strips; and the remainder of 800 ml mixed with water to give a dilution of 5 parts buffer to one part water, for use in the electrophoresis chamber.

(c) Preparation of Strips

The strips used were S & S Cellulose Acetate Strips supplied by Consolidated Laboratories (Canada) Ltd., size 2.5 x 12 cm. A normal

run involved the analysis of two plasma samples, each one in duplicate, so that a total of four strips were used. Each strip was marked, while still dry, with the number of the sample to be applied and the number of the run; two small x's were used to mark the point of application of the sample; all marks were made lightly with a ball-point pen. Buffer impregnation was accomplished by first floating the strips on the surface of the full-strength buffer and finally immersing them completely. At the same time, four wicks of filter paper were similarly soaked in full-strength buffer; these were subsequently used to line the strip-holders in the chamber and served to maintain contact between the strips and the buffer in the chamber.

(d) Preparation of Samples

The plasma samples to be so analyzed were removed from the freezer and thawed at room temperature. At first, analyses were carried out on the undiluted plasma; but I found that the high protein content of the plasma (to be considered later) led to densely-stained patterns with considerable loss of definition. After several trials, satisfactory results were achieved when the plasma was diluted to one-half its original concentration; one-half milliliter serological pipettes were used for this purpose. As is standard procedure, the samples were applied to the strips outside of the chamber. Each strip in turn was carefully removed from the buffer, using smooth-tipped Gelman forceps, and blotted lightly between two sheets of bibulous paper to remove excess surface moisture. The following operations were carried out as quickly as possible in order to avoid drying out the strip.

Fig. 6. Shandon Universal Electrophoresis Tank with cellulose acetate strips in position.

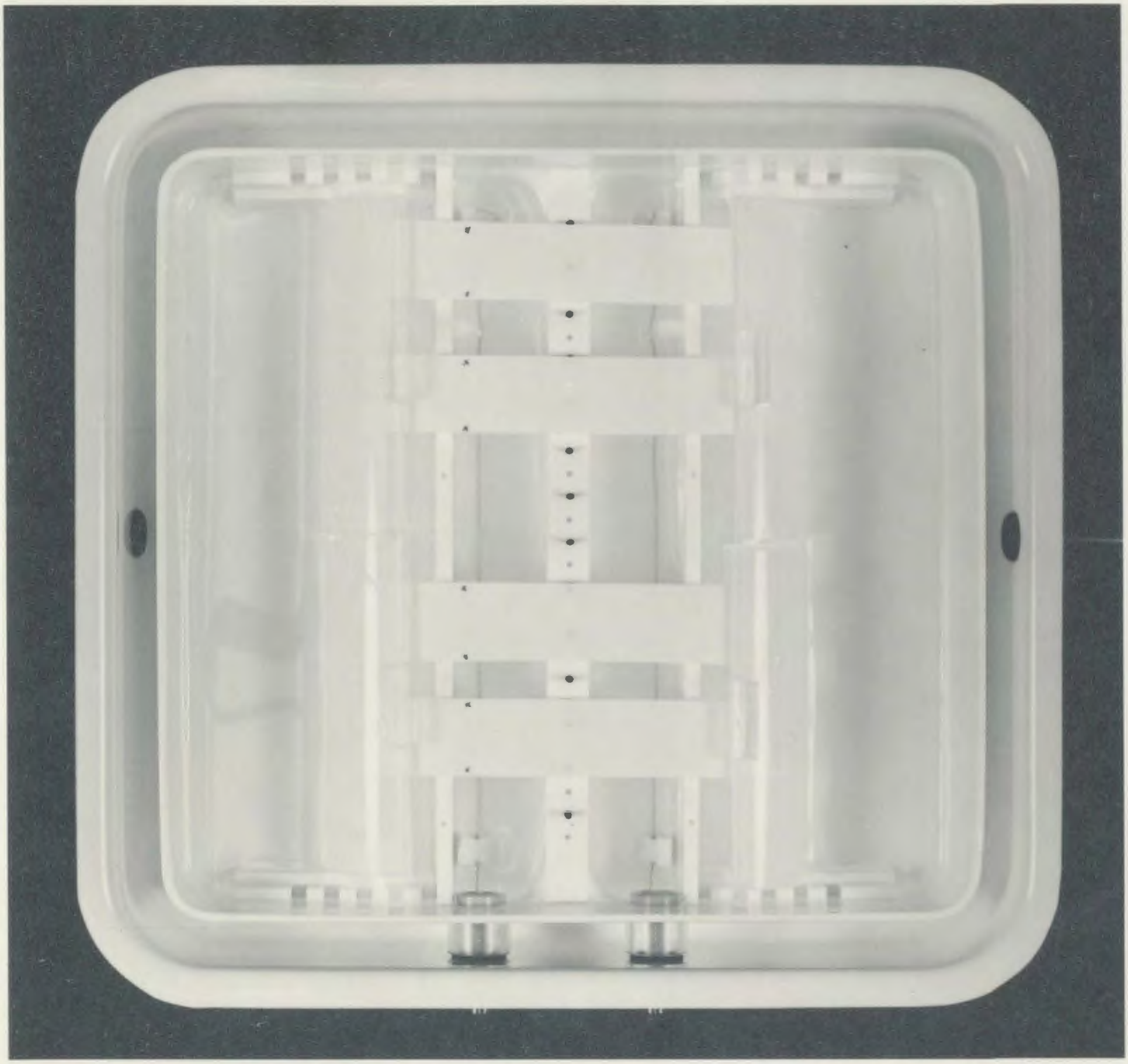
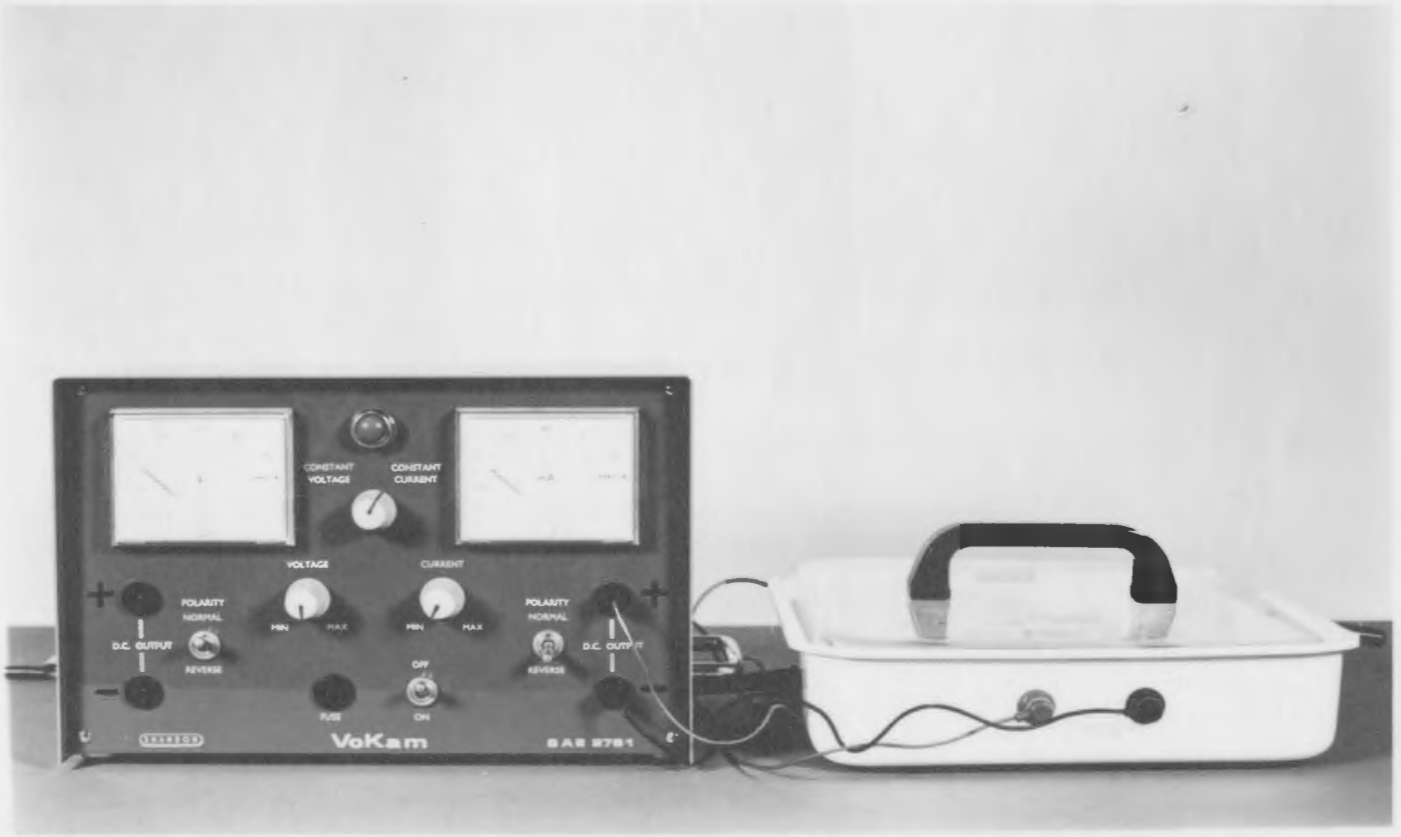


Fig. 7. Shandon Universal Electrophoresis Tank connected to the Shandon Vokam direct current power supply unit.


CONSTANT VOLTAGE
CONSTANT CURRENT
VOLTAGE
CURRENT
POLARITY NORMAL
REVERSE
D.C. OUTPUT
MIN
MAX
FUSE
OFF
ON
VoKam

(e) Application of Sample to Strip

A two microliter sample of plasma was withdrawn from the vial, using a Drummond "Microcap" disposable micropipette (Consolidated Laboratories). This was transferred to a Gelman electrophoresis sample applicator, this simple device consisting of two fine parallel stainless steel wires fastened at each end to a handle, the sample being held between the wires by surface tension. The sample was then applied to the strip at the premarked point about three centimeters from what was to be the cathode end of the strip. The plasma was applied in a uniform line at right angles to the long axis of the strip. The strip was then positioned in the chamber and carefully fixed between the strip-holders to prevent sagging. Following the placing of each strip, the cover of the chamber was replaced in order to avoid loss of moisture.

(f) The Electrophoretic Run

The chamber used for all of the analyses was the Shandon Universal electrophoresis tank (Fig. 6). A constant current (rather than constant voltage) was used for all runs, the constant current being supplied by a Shandon Vokam d.c. power supply unit (Fig. 7). A current of one mA per strip was used; during a run of one hour and thirty minutes, the voltage would fall from an initial value of about 200 to 220 volts to a final reading of 130 to 140 volts. The following information was recorded on a work sheet at the time of each run: the date; the number of the run; the numbers of the samples; the temperature and pH of the buffer in the chamber; the current and the initial and final voltages, as read from the power supply; the duration of the run.

(g) Staining and Drying of Strips

The protein-specific stain used for all strips was Ponceau S (Consolidated Laboratories (Canada) Ltd.) at a concentration of 0.2% in a 3% aqueous solution of trichloroacetic acid. Each strip was floated on the surface of the stain solution immediately after removal from the electrophoresis chamber. As soon as the bands began to stain, the strip was submerged completely. The minimum staining time ranged from five to ten minutes, but the strips were sometimes left in the stain overnight, as is common practice, without any alteration of the bands. Background stain was removed by washing the strip for about two minutes in each of three successive trays of 5% aqueous acetic acid. Drying, when necessary, was accomplished by suspending the strip vertically at 50°C for 30 minutes, the drying oven being adjusted for maximum ventilation to eliminate acetic acid fumes.

(h) Clearing of Strips

In order to prepare the strips for evaluation by densitometric scanning, it was necessary to clear them, that is, to provide a transparent background for the electrophoresis patterns. Two methods of accomplishing this were attempted.

The first method involved soaking the strip in an oil of suitable refractive index, in this case Whitemor Oil no. 120. The dry strip was floated on the surface of the oil until soaked from below and then immersed completely. By this method the strips became transparent almost immediately.

In the second method, the strip was treated immediately after washing, with no drying. The strip was removed from the final acetic acid bath, drained thoroughly and immersed in 95% ethanol for one minute, with continuous agitation. Following another thorough draining, the strip was transferred to a tray of clearing solution prepared as follows:

Acetic acid, glacial	30% by volume
Ethanol, 95%	68% " "
Glycerol	2% " "

In this solution the strip was agitated continuously for one minute and occasionally for a period of four minutes (total time: five minutes). A glass plate measuring two inches by six inches was placed on the bottom of the tray and the strip was positioned over it. The plate and the strip were then removed together, drained briefly and held at an angle with the lower end of the plate resting on a pad of absorbent tissue. In order to hasten the drying of the strip, surface moisture was gently wiped from the strip on to an absorbent pad following which the strip was allowed to dry in a horizontal position at room temperature for one minute.

Both ends of the strip were fastened to the glass plate, using cellophane tape, and the strip was dried at 60° to 70°C for 20 minutes with maximum ventilation. After allowing the plate to cool, the tape and the strip were carefully peeled from the plate and the tape then cut off the strip.

I consider the first method, using the clearing oil, to be the more satisfactory one. It has the advantages of speed and simplicity and the presence of the oil in the strip adds considerably to the pliability and tensile strength of the strip during subsequent handling and storage. The strip remains completely transparent as long as sufficient oil is present.

The second method, however, has the advantage of producing a dry cleared strip but I feel that this is offset by the length and tediousness of the method. Also, extreme care must be taken in wiping away surface moisture as the wet strip is easily stretched and distorted, resulting in a corresponding distortion of the electrophoretic pattern. I found, further, that some of the strips cleared in this way were not completely transparent; rather, they had a slightly cloudy appearance over all or part of their surface.

(i) Storage of Strips

The completed electrophoresis strips, whether cleared or uncleared, were stored between glassine sheets designed for the storage of 35 millimeter photographic negatives.

In addition to the *Illex* blood plasma thus studied, several samples of normal human blood sera were also subjected to electrophoretic analyses. Human serum was chosen because it is one of the most frequently analyzed protein-containing fluids (Moore, 1959). Indeed, such analyses are performed as part of the daily routine of nearly every clinical

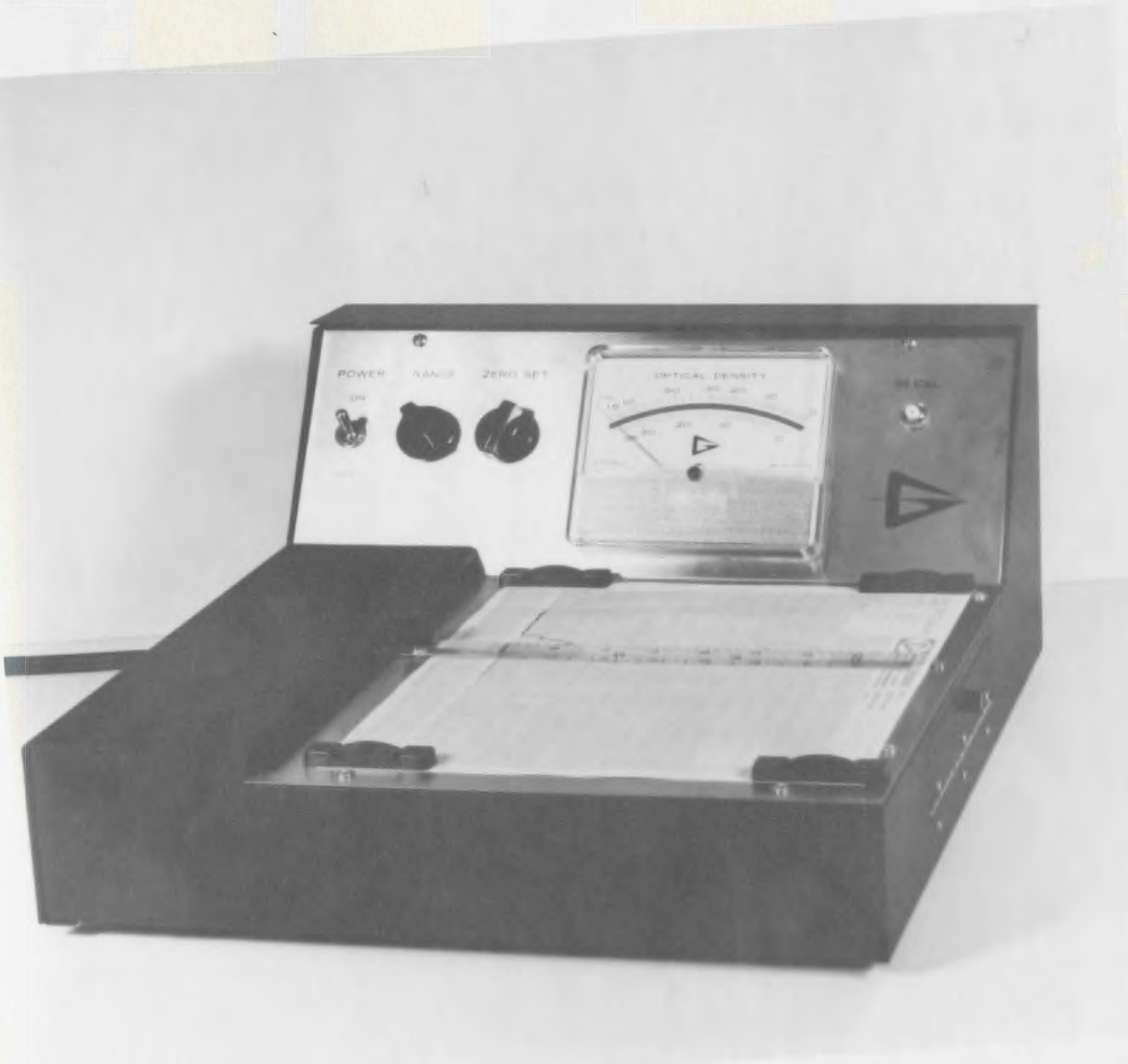
laboratory. As its characteristic pattern has thus become so well known, it was felt to be a logical choice to serve as a periodic check not only on the equipment and the technique, but also to serve as a basis for comparison of such a factor as the rate of migration of the protein fractions.

(j) Evaluation of the Electrophoretic Patterns

Evaluation of the separated protein fractions was achieved by densitometric scanning of the cleared strip. This was carried out manually using the Gelman Electrophoresis Scanner, no. 39301-1 (Fig. 8), which measures the absorbed dye intensity of the protein fractions and simplifies the plotting of results.

The scanner functions on the principle of light transmission through a sample strip of varying dye intensity. A defined light beam passes through the sample and a contrasting color filter enables the instrument to distinguish small differences in dye absorption. The transmitted light is redefined before striking the light-sensitive photoconductive cell. The output signal from the cell, which is a measure of the percentage of light transmitted through the strip, is then converted to optical density units as indicated on the meter. A plotting rule on the deck of the instrument is mechanically coupled to the photohead mechanism, enabling the operator to record instantly, on the graph paper beneath the plotting scale, each successive value of optical density as read from the meter. Scherr (1961) has shown that, when cellulose acetate is used as a medium, all serum protein components stained with Ponceau S take up the dye only in relation to their respective concentrations.

Fig. 8. Gelman Electrophoresis Scanner.


POWER
ON
RANGE
ZERO SET
OPTICAL DENSITY
IN CAL

The cleared strip is placed between two glass slides in a slide holder and inserted into the instrument by way of a door at the side (Fig. 8). The plotting rule is then moved slowly toward the operator and the meter readings are plotted on the graph paper, resulting in a series of peaks, each of which corresponds to a stained band on the strip.

Some difficulty was encountered in using the Gelman scanner, due to rapid fluctuations in the level of the line voltage, sometimes as large as three or four volts. These fluctuations caused unstable meter readings, making it difficult at times to plot accurate optical density values. Such variations in the line voltage are common throughout the building and indeed throughout the city of St. John's. The instrument, though equipped with a small constant voltage transformer, was apparently unable to compensate for the rapid changes. A larger transformer unit, which might have solved the problem, was not available.

A Gelman compensating polar planimeter, Model 39231, was used to measure the area under the peaks of the graphic record. An attempt was then made to express the area under each peak as a percentage of the total area under all the peaks and, using the total protein concentration already determined, to estimate the concentration of the protein fraction represented by each peak.

In view of the difficulties experienced with the manual scanning technique, I decided, with permission, to run several strips through an automatic scanner located in the Biology Department on the main campus.

This instrument is a Densicord recording electrophoresis densitometer (Model 542) (Consolidated Laboratories Ltd.), equipped with a Cellulose Acetate Drive (no. 5073) and coupled with an Integraph integrator (Model 49). As the densitometer automatically scans the strip and plots a graph of optical density, the integrator measures the area under each peak and records the results as a continuous record beneath the graph.

The usefulness of the Integraph was limited, however, by the fact that only one scanning speed was possible. The implications of this will be discussed later.

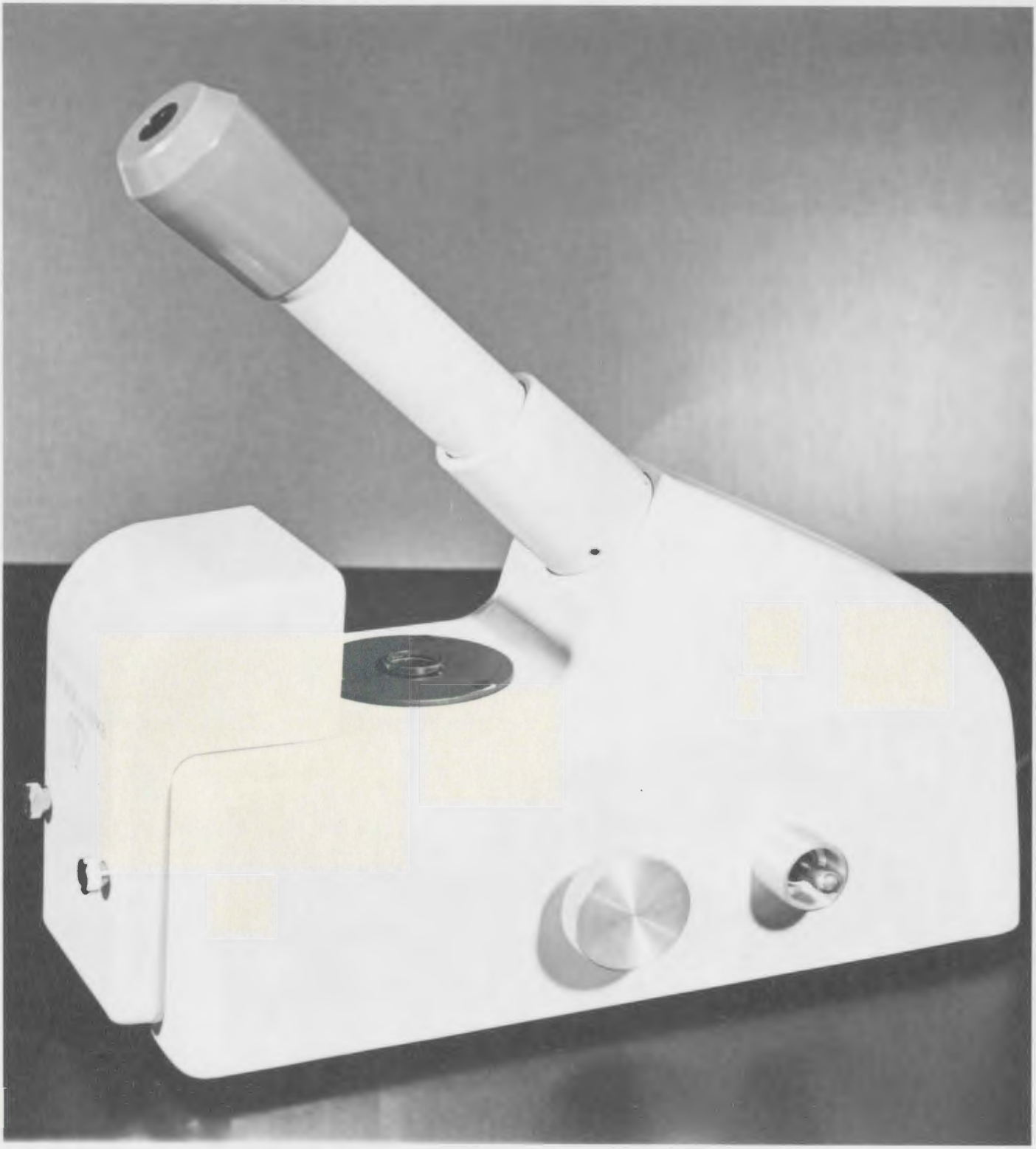
2. Determination of Total Protein

The total protein content of the *Illex* blood plasma was determined refractometrically, by the use of a Bausch & Lomb serum protein meter (no. 33-45-87).

(a) Basic Principle

The principle of measuring serum proteins refractometrically was introduced by A. Strubell in 1900. It is first necessary to derive an equation relating the serum protein concentration to the refractive index of the serum, as measured by a refractometer. In order to do this, a series of diluted sera is prepared from a standard, the protein concentration of which has been determined by one of the long-established methods, such as the biuret technique (Gornall, Bardawill and David, 1949). The refractive indices of these sera are measured, the refractometer values being plotted against the serum protein concentrations

Fig. 9. Bausch & Lomb serum protein meter.



of the solutions. A regression line is calculated statistically and its equation is used in the determination of the protein in the serum being examined. Once the equation has been derived, the technique becomes routine. The refractive index of distilled water is subtracted from the refractive index of the test serum, the difference being substituted in the equation to give the protein concentration (Sunderman, 1944).

(b) Analysis of the Plasma

In practice, most of these steps are eliminated by the serum protein meter. This instrument is a refractometer, having two scales which are read through one eyepiece (Fig. 9). The first of these scales is in refractive index difference from water, to be used in an equation of the operator's own choice. The second scale gives direct readings of serum protein, over a range of 0 to 12 g/100 ml, the scale having been computed according to the formula:

$$Pr = 524(RI_{diff.}) - 1.10$$

where Pr is the serum protein in g/100 ml and $RI_{diff.}$ is the difference between the refractive index of the serum and that of distilled water.

The direct reading serum protein scale was used in all determinations and it proved to be sufficiently accurate for the purpose of the present work.

The meter must first be standardized or zero-set, using distilled water. A medicine dropper with a curved tip was used to place the water

sample in the cuvette located on the top of the instrument, the index line then being adjusted to coincide with the "H_2O" line on the protein scale. The water was then removed and the cuvette carefully dried.

A sample of *Illex* plasma was placed in the cuvette and its protein concentration read at the point where the index line crossed the scale. It was discovered immediately that this concentration lay beyond the range of the meter, that is, it was greater than 12 g/100 ml, and that dilution of the plasma was therefore necessary. A dilution of 1:1 was found to be satisfactory and was carried out immediately prior to examination, using ½-ml serological pipettes. It was consequently necessary to multiply the protein scale reading by two in order to obtain the value for undiluted plasma.

The refractive index of water and of serum protein decreases approximately one unit in the fourth decimal place for an increase in temperature of one degree centigrade. Some type of temperature compensation is therefore necessary when the refractive indices of liquids are being measured. As the meter has no temperature compensation control, the distilled water and the plasma samples were allowed to attain room temperature before carrying out the determination. All values, therefore, were obtained at a temperature of approximately 22°C.

(c) Checking the Meter

As a check on the meter, samples of a standard protein solution (no. 7585, Hartman-Leddon Company, Philadelphia, Pennsylvania) were examined periodically. (Total protein content of the standard solution: 4.85 g/100 ml)*

*See Addendum 2, page 82.

3. Immunodiffusion

(a) Production of anti-*Illex* antiserum

Several plasma samples from different female specimens of *Illex* were pooled in order to prepare a uniform antigenic solution of sufficient volume to maintain a series of injections. The plasma was injected into a rabbit subcutaneously at the top of the head, between the ears, using a hypodermic needle and syringe. The schedule of injections was as follows: three daily injections of 0.5 ml each; after a four-day interval, two daily injections of 0.5 ml each; after a thirteen day interval, three daily injections, the first two of 0.5 ml each and the last of 1.0 ml.

The rabbit was bled approximately six weeks after the final injection, 37 cc of blood being allowed to drip freely from the posterior marginal vein of the left ear. This blood was allowed to clot and left to stand overnight at 5°C. The serum was decanted carefully and the remaining blood was centrifuged at 1750 rpm in order to obtain a further quantity of serum by shrinking the clot. The serum, which is hereafter called antiserum, was divided into aliquots of one to two milliliters and stored in screw-topped glass vials at -20°C.

(b) Diffusion tests

Gel diffusion tests using Ouchterlony-type agar gel plates, as described in the Introduction, were attempted on two different occasions. It was then decided to carry out further diffusion tests in a cellulose acetate medium, using the method described by Consden and Kohn (1959).

Cellulose acetate membranes have several advantages over agar gel as a medium for immunodiffusion: the uniformity of cellulose acetate is excellent; its speed and sensitivity are superior to gel media, making possible the use of very small volumes of sera; it provides a durable, easily-stored permanent record; and there is continuity without the interruption of wells which frequently interfere with the precipitation patterns.

For the purpose of these tests, portions of convenient size were cut from cellulose acetate electrophoresis strips. The points for application of the antigen and antiserum were marked on the strip with a ball-point pen, the mark being identified by the number of the *Illex* plasma and by the letter "A" for antiserum, respectively. The loci thus designated were placed approximately one centimeter apart.

The strip was impregnated with barbitone buffer (Owen, 1956), as for electrophorsis, then blotted to remove surface moisture. The strip was then placed on a plastic "Pin-cushion" support inside a closed container to prevent drying out while the sera were being applied.

The *Illex* plasma sample, after being diluted 1:1 as for electrophoresis, was deposited at the pre-marked point on the strip, using a Drummond "Microcap" two-microliter micropipette. The same quantity of rabbit anti-*Illex* antiserum was similarly applied at its pre-marked point. The samples were permitted to soak into the strip, the cover of the container being replaced immediately after application. The strip was then completely submerged in Whitemor Oil no. 120 and left for 24 hours,

during which time diffusion of the samples occurred.

Following the diffusion period, the strip was removed from the oil and blotted. Traces of oil were removed by washing the strip in detergent solution for thirty seconds and rinsing thoroughly. The strip was then placed in another barbitone buffer solution (pH 8.6) for at least three to four hours, and sometimes overnight.

Staining of the precipitin pattern was accomplished with Ponceau S (0.2% in 3% aqueous trichloroacetic acid), just as for the electrophoresis patterns. The strips were not cleared.

The antiserum was tested against single plasma samples from either male or female squid, as well as against both sexes simultaneously and against the same sex from different times of collection, the latter being an attempt to detect any differences due to sexual maturity.

As already indicated, this aspect of the research was undertaken as a preliminary qualitative study only. No attempt was made to measure the quantity of antigen-antibody complex produced.

RESULTS

1. General Observations

The volume of blood obtained from a single specimen of *Illex* ranged from approximately one-third of one cubic centimeter to about 8 cc; more than 80% of the samples, however, were between 1 cc and 6 cc in volume. The volume of samples obtained depended upon certain factors already described. Over 460 squid were bled in this study. Only 412 samples were kept and stored, however, since all samples of less than 1 cc were discarded.

The blood of *Illex* is a thin liquid, though slightly more viscous than water. Its color depends upon the degree of oxygenation of the respiratory pigment, hemocyanin. In the venous, de-oxygenated state in which my samples were taken, the blood has a bluish white color. In the arterial circulation in the living animal, the blood has been observed to range through pale blue, light blue and pastel blue. By exposing the blood to air and shaking the container thereby placing oxygen in solution, it is possible to cause it to take on a deep blue color, but such a shade of blue has not been seen in vivo. Colors here follow the coding by Kornerup and Wanscher, 1961.

2. Determination of Total Protein

The results of this aspect of the research are presented first, so that they may be applied in conjunction with those of the electrophoretic study.

The total protein values of the *Illex* blood plasma samples examined by use of the serum protein meter ranged from 11.30 to 14.50 g/100 ml of plasma. The average value obtained was 12.76 g/100 ml of plasma. Both of these extreme values (maximum and minimum) represent plasma samples from female specimens, the average for all females being 12.80 g/100 ml of plasma. No trend was evident to indicate any sex-related differences in the protein content of the plasma, for the value obtained for the single male *Illex* plasma examined was 12.60 g/100 ml. Two females taken at quite different times in the season (August and October) both had plasma protein values at the lower end of the range.

3. Electrophoresis

Each and every electrophoresis pattern obtained with *Illex* blood plasma, regardless of sex or time of capture, consisted of two,and only two, components or protein fractions, one of which stained much more intensely than the other. Both fractions migrated from the cathode toward the anode, the lighter component (which I have designated as no. 1) moving at a slightly higher rate than the darker one (no. 2).

When normal human blood serum was run at the same time on an adjacent strip, it was shown that component no. 1 migrated at approximately the same rate as the human α-globulin fraction asnd that the rate of migration of component no. 2 was slightly higher than that of human β-globulin.

In Figure 10 is shown a photograph of one electrophoretic

Fig. 10. Electrophoretic separation of the proteins of the blood of *Illex illecebrosus illecebrosus*, on cellulose acetate, following a run of one hour at pH 8.6. Current: constant at 1 mA per strip. Voltage: initial, 190 V; final, 140 V. Temperature inside the chamber, 22°C. The two protein fractions have been numbered arbitrarily. Stained with Ponceau S. Cleared strip was photographed by transmitted light.

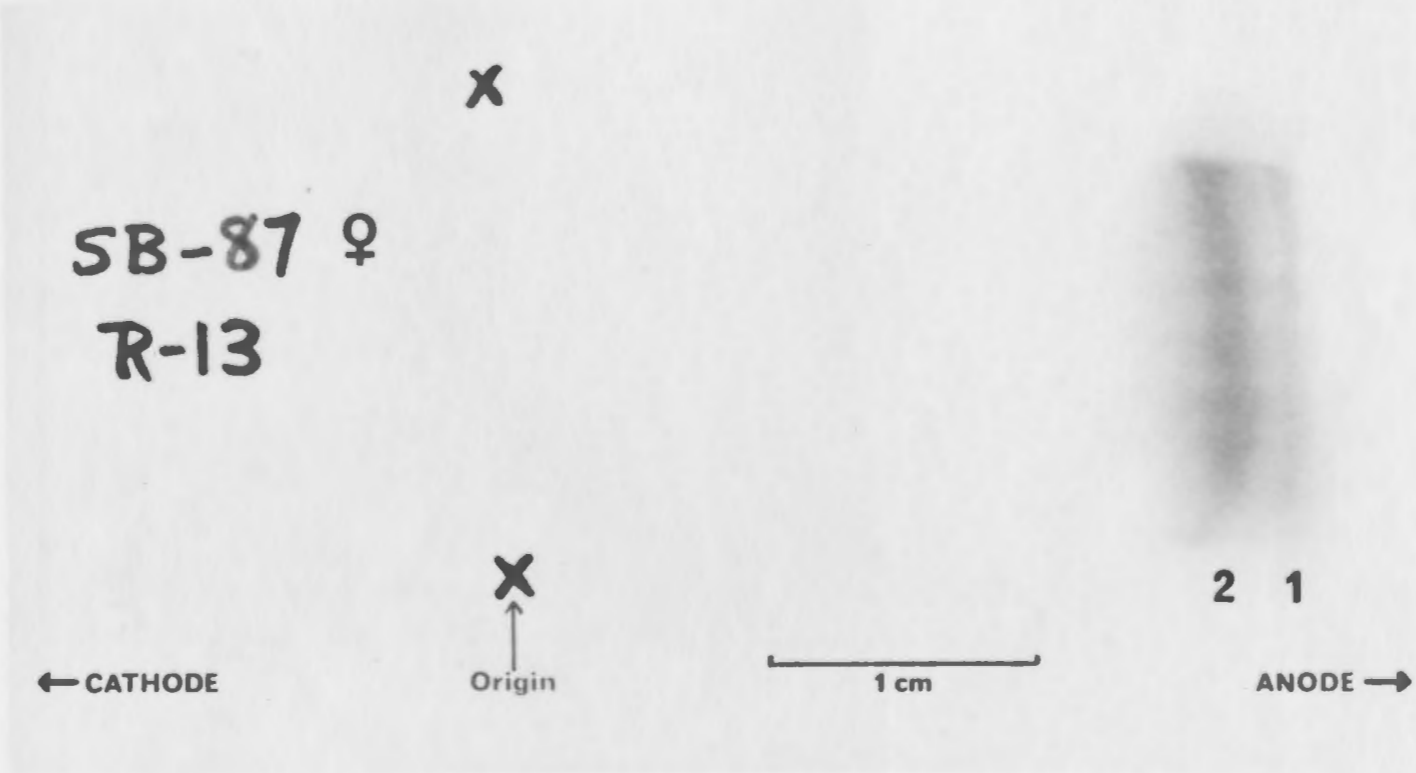
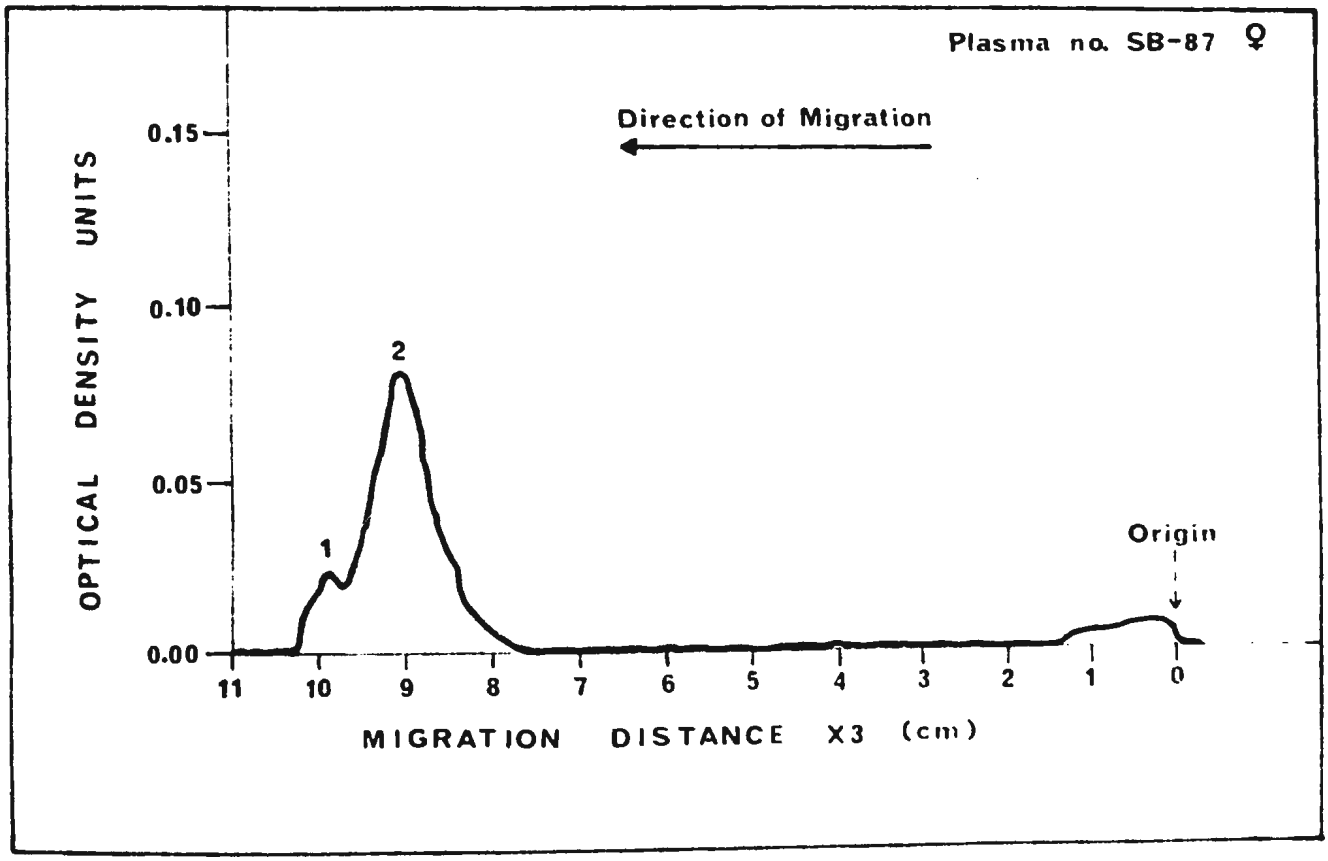

SB-87 ♀
R-13
2 1
CATHODE
Origin
1 cm
ANODE

Fig. 11. Densitometric graph of the electrophoretic pattern of the blood plasma of *Illex illecebrosus illecebrosus*, as shown in Fig. 10. The numbered peaks correspond to the numbered protein fractions of that pattern.


Plasma no. SB-87 ♀
Direction of Migration
OPTICAL DENSITY UNITS
0.15
0.10
0.05
0.00
1
2
Origin
11
10
9
8
7
6
5
4
3
2
1
0
MIGRATION DISTANCE X3 (cm)

separation of the two protein fractions. In all trials, as noted above, every case of the dozens made exhibited like results and the pattern reported in Figure 10 is true for all electrophoresis trial results. Separation of the components is clearly seen and there is very little "tailing-back" at the ends of the bands.[1]

A densitometric graphic record of an electrophoresis pattern is shown in Figure 11. For the sake of continuity, the same *Illex* plasma sample, no. SB-87, has been used as an example. As noted earlier, diluted plasma was used, which has resulted in low optical density values. However, the relative optical densities are unchanged from what would have been demonstrated if whole, undiluted plasma had been used. It will be noted that the plotting scale of the densitometer has produced a three-fold linear expansion in the migration distances from point of origin, when compared with the linear distances represented in Figure 10.

The areas under the peaks in Figure 11 were measured to be:

Component no. 1 . . . . . 0.07 sq in

Component no. 2 . . . . . 0.45 sq in

Total area . . . . . 0.52 sq in

By expressing each component as a percentage of the total and by relating this to the average value for total protein of 12.76 g/100 ml

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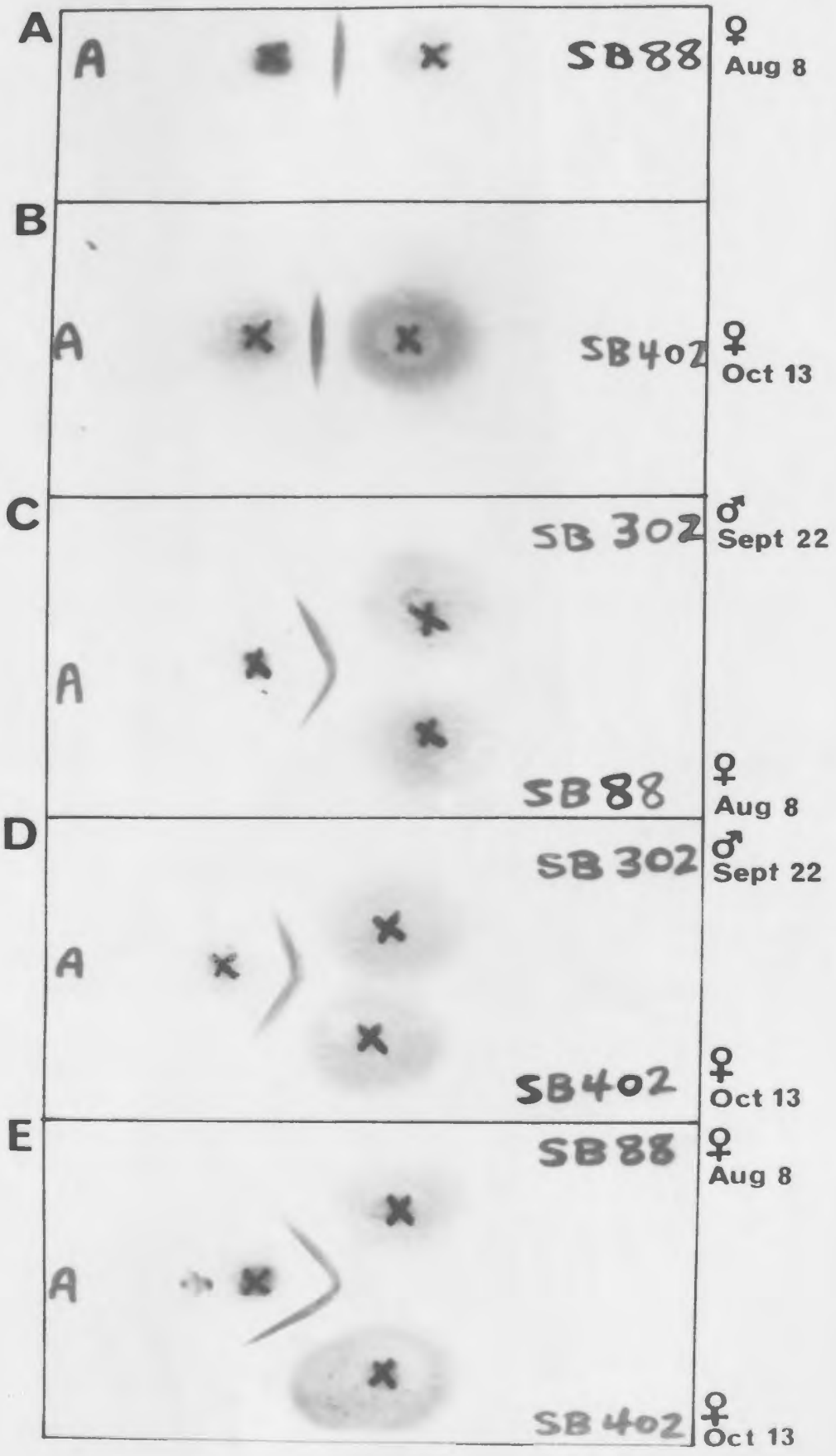
[1]The plasma in this case was no. SB-87, female, from South Arm, Holyrood Bay. The constant current was one milliampere per strip (total 4 mA): the initial and final voltages were 190 volts and 140 volts, respectively; the temperature in the chamber was 22°C, and the duration of the run was one hour.

Fig. 12. Immuno-diffusion on cellulose acetate membranes, involving blood plasmas of three specimens of *Ilex illecebrosus illecebrosus* and rabbit anti-*Ilex* antiserum (A), at pH 8.6 for 24 hours. Antigen and antiserum placed 1 cm apart. Stained with Ponceau S. Uncleared membranes photographed by transmitted light.


A
A
SB88
♀
Aug 8
B
A
SB402
♀
Oct 13
C
SB 302
♂
Sept 22
A
SB88
♀
Aug 8
D
SB 302
♂
Sept 22
A
SB402
♀
Oct 13
E
SB88
♀
Aug 8
A
SB 402
♀
Oct 13

of serum, the following values were obtained:

Component no. 1	13.5%	1.72 g/100 ml
Component no. 2	86.5%	11.04 g/100 ml

4. Immunodiffusion

The results obtained from the two-dimensional diffusion tests using the Ouchterlony agar gel plate method were, for all practical purposes, negative. Most of the plates gave no evidence of any precipitin reaction. A few, however, showed slight cloudiness in the area between the antigen and antibody, but no distinct configuration which is characteristic of antigen-antibody complex.

Much more encouraging results were obtained from the tests carried out in cellulose acetate. A single band of precipitate, made visible by the Ponceau S stain, appeared in each case. Whenever two antigens were used simultaneously, as in the case of introduced male and female *Illex* plasma samples, the two single bands (one caused by each antigen) merged into one continuous band.

Figure 12 shows photographs of five representative tests, covering a variety of cases. The first two (A and B) involve antiserum and a single *Illex* plasma sample, while C, D and E were double tests, in which two plasma samples were tested simultaneously against the antiserum. In each case, as already stated, a single band of antigen-antibody complex has been formed between the *Illex* plasma and the rabbit anti-*Illex* antiserum, which is designated by the letter "A". To the right of the test strips are data regarding the sex and time of collection of each

Illex plasma sample. In the double tests shown in C, D and E, the band of precipitate is seen as a continuous arc in the region between the antibody and the two antigens.

DISCUSSION

"...the value of serum protein analysis ... does not depend entirely on the possibility of obtaining accurate absolute measurements of the various fractions present. What is required is a reasonably reproducible index of these fractions relative to each other or to the total amount of protein present. Changes in these indices then become of significance, even though the extent of these changes cannot be computed in precise terms." -- Owen, 1956.

The electrophoretic analyses were regarded, from the beginning, as constituting the major portion of this research. The greatest proportion of working time was spent on this aspect of the study and it has in turn, I believe, yielded the most important results.

It is a well established fact (Prosser and Brown, 1961; Meglitsch, 1967) that in the hemolymph or blood (as the case may be) of molluscs, nearly all of the protein is in conjugated form in the respiratory pigment. In most molluscs, the respiratory pigment is hemocyanin, and it has been found to include over 90% of the blood protein. In view of the high protein content recorded for *Illex*, it seems certain that this conjugated protein will be represented in any electrophoretic separation.

Each electrophoretic separation of the plasma proteins of the blood of *Illex* has produced two very closely allied components. According to the densitometric data, the two components are, in fact, incompletely separated. At least one of these must be the proteinaceous portion of the hemocyanin.

In each immuno-diffusion test, a single band of antigen-antibody precipitate has appeared, indicating the presence of at least one antigenic

component in the *Illex* plasma. In view of the electrophoretic data achieved, at least two antigenic components would be expected. A possible explanation of the observed situation in contrast to what would be expected, is that both of the protein fractions appearing in the electrophoretic pattern may be attributed to hemocyanin. Woods, Paulsen, Engle and Pert (1958) call attention to the fact that, in alkaline media hemocyanins usually dissociate into nonidentical constituents. In this study, the analyses of *Illex* sera were made in alkaline media. In the only other electrophoretic analysis of squid blood reported to date, Woods, et al. (1958) report that the blood plasma of *Loligo pealei* LeSueur yielded two major components of moderate mobility. Both of these components they consider to be due to the hemocyanin. Sera of several other invertebrates analysed at the same time similarly gave more than one protein fraction of high concentration, for which the same explanation is offered. Prosser and Brown (1961) state that the hemocyanin of the horseshoe crab *Limulus polyphemus* (L.) separates electrophoretically into two components.

It is interesting to note that, using a slightly different technique, the present analyses have produced a similar pattern to that obtained in the study by Woods, et al involving such a closely related animal. The two protein fractions they reported occur very closely together and have migrated away from the cathode toward the anode, as was the case with the *Illex* plasma. The authors state that, within a given species, differences in age, sex, or stages of the molt cycle (in crustaceans) produced no significant difference in the electrophoretic patterns.

In a direct comparison between the electrophoretic migration rates of the proteins in *Illex* plasma and normal human serum, we have seen that component no. 1 corresponds in mobility with human α-globulin, with component no. 2 slightly less mobile. Favour (1958) analyzed electrophoretically the hemolymph serum of the crab, *Cancer pagurus* L. together with the sera of several other animals. The results are presented as densitometric graphs. As *Cancer* was the only species of invertebrate in the group so studied, the peaks recorded were labelled arbitrarily by use of the standard Greek symbols common to the vertebrate protein fractions to which they most nearly corresponded. The α-globulin peak proved to be a major component, leading Favour to regard it as representing the hemocyanin of the serum.

Therefore, it is not unreasonable to assume that *Illex* component no. 1 is hemocyanin, based on mobility characteristics when viewed in light of the findings of Favour (1958). The exact nature of component no. 2, still unknown, may be another conjugated protein involving the hemocyanin complex or a part of same, this assumption based on findings referred to earlier here by Woods, et al.(1958) for *L. pealei*.

The densitometric graph presented as an example in Figure 11 was chosen, as noted earlier, for the sake of continuity, being representative of the same *Illex* blood plasma sample which is represented in Figure 10. The clear separation of the protein fractions on the electrophoresis record, together with the virtual absence of "tailing", has resulted in a densitometric tracing of comparatively high quality, in which the two peaks, representing the two fractions, are clearly visible.

Near the point of origin on this figure, the tracing describes a small peak which at first glance might be interpreted as representing a third protein fraction in the plasma. However, it is much more probable that the stained region on the electrophoretic strip which gives rise to this peak on the graph is due rather to the presence of a small quantity of plasma protein which, due to some unknown factor related to the method of applying the sample to the strip, has remained behind at or very near the point of application. This stained region is less obvious on the strip than the densitometric graph would indicate. However, such a region did appear in virtually every electrophoretic record. Its position was always the same, regardless of the duration of the run, that is, it did not migrate. Favour (1958) reports a similar phenomenon, which he calls a "technical residue at the point of application", in records of the electrophoresis of the blood serum of the crab *Cancer pagurus*.

The presence of this technical residue, including a small fraction of the protein content of the plasma, makes it necessary to point out that the values calculated in the preceding section are percentages of the protein represented by the total area under the two peaks of the graph, numbered 1 and 2.

Brief mention was made earlier of attempts to obtain densitometric records from the electrophoresis strips by the use of a Densicord recording electrophoresis densitometer coupled with an Integraph integrator. These attempts were unsuccessful, due to the fact that the single scanning speed possible was too high. The two plasma protein fractions of the blood of

Illex, which lay very close together even after runs of two hours or more, were scanned so quickly that the peaks of the graph were virtually fused in one. The separation between the peaks was barely noticeable. This rendered the automatic tracing much less useful than that obtained by use of the Gelman scanner. The adjustment of the Densicord instrument permitting lower scanning speeds by the use of proper accessories will permit more sensitive examinations of electrophoresis patterns.

The study of the total serum protein content of the blood plasma of *Illex* was intended,as indicated earlier, to be a minor part of this research programme. However, a brief discussion of the accuracy to be expected when using the refractometric technique may be in order at this point.

Sunderman (1944), in discussing refractometric techniques, mentions three factors which might alter the refractometric values in relation to protein concentration. The first of these is a high concentration of bilirubin present in the serum. Since bilirubin is a metabolic breakdown product of hemoglobin, a compound not present in cephalopods, it is certainly not a factor in this case. The second possible factor is a high level of lipid, which there is no reason to suspect here.

Rubini and Wolf (1957) have carried out refractometric determinations of total solids in serum and have found that certain factors formerly believed to lower the accuracy of refractometric determination of total protein have had no such effect on their measurements. Their data do not

support the view that the refractometric determination of total protein should be adversely affected by variations in the albumin-globulin ratio. Furthermore, they state that various protein fractions in saline do not differ appreciably from serum in the specific instrumental increment of the refractometer. They conclude, therefore, that as the non-protein solid content is constant in plasma, and because it contributes only a small fraction of the refractivity, empirical relations between the refractive index (or the refractive index difference) and total protein of serum should prove sufficiently accurate for many purposes.

The values obtained for total protein in the blood of *Illex* are high when compared with typical values for vertebrates. The serum protein of man, for example, has been measured to range from 5.38 to 9.40 g/100 ml of serum (Dittmer, 1961). These high values in *Illex* are, of course, due to high concentrations of hemocyanin present in the plasma. The presence of large quantities of respiratory pigment in the bloods of cephalopods may be related to the high metabolic rates of these animals and the low oxygen-combining power of hemocyanin. The oxygen consumption of squids swimming freely in an aquarium has been reported by Redfield and Goodkind (1929) to be approximately 600 ml/kg of body weight per hour, which is about three times the basal metabolic rate in man.

As already noted in the Introduction, the total protein in the blood of the squid, *Loligo forbesi* (Steenstrup) has been reported by Robertson (1949) to be 14.97 g/100 ml of plasma, which is just beyond the upper limit of the range of values obtained here for *Illex*. Robertson's

value for the cuttlefish, *Sepia officinalis*, was considerably lower (6.76 g/100 ml), while that for the octopod, *Eledone cirrhosa*, lay between these two (10.5 g/100 ml). Robertson notes that the higher organization and activity of these three cephalopods appears to be related to high plasma protein contents, which may be necessary to meet high respiratory demands. Such an assumption would seem quite reasonable, since within the group studied, *Sepia* has a lower plasma protein content than *Loligo;* this would be in keeping with the fact that *Sepia* leads a much less active existence, remaining nearly stationary on or in the ocean bottom for long periods of time, while *Loligo*, like *Illex*, spends much time swimming actively in the open water.

Akimushkin (1963) notes that almost all squids are excellent swimmers, being able to move more quickly than any pelagic fish of comparable size. The Ommastrephidae, Loliginidae, Gonatidae and Onychoteuthidae, he states, are especially swift. Fields (1965) writes that *Loligo opalescens* Berry has been observed swimming at speeds of from five to eight miles per hour. Some squids are capable of such speeds that they are able to leave the water and glide through the air for considerable distances, in a manner similar to that of "flying" fish; distances of 50-60 yards have been reported (Zuev, G. V., personal communication to F. A. Aldrich). Squids hunt for food both day and night, their prey ranging from plankton to fast, powerful swimming fish. They make rapid attacks in the open water and usually kill by biting the fish at the back of the neck, though they sometimes attack the belly of very large prey such as a dogfish. Squids

are highly aggressive predators and have been observed to kill a number of fish in rapid succession without eating them, as if for sport (Lane, 1960).

Generally, cuttlefish are not as swift as squids. Their oval bodies are not as efficient in cleaving the water as are the torpedo-shaped bodies of squids. Cuttles normally swim by means of comparatively slow jets from their funnels, probably aided by undulations of the fins. They prey for food upon small crabs, shrimps, prawns and small fish, feeding only during daylight hours (Lane, 1960). Though cuttlefish sometimes seek prey in the open water, they most often feed on animals living on or near the sea bottom. They seem to prefer a sandy or silty bottom and frequently lie partially buried in the sand, waiting for prey to pass nearby. The exposed parts of the body can be matched in colour to almost any bottom (Akimushkin, 1963).

The vast majority of octopods are bottom dwellers. They can move rapidly when necessary, but are generally sessile creatures (Akimushkin, 1963). Lane (1960) considers that octopods are generally not such fast swimmers as squids and cuttlefish, while at the same time noting that *Octopus vulgaris* Cuvier has been reported to swim at about four miles per hour while on migration. *Octopus vulgaris* captures prey both by lying in wait and by hunting, mostly at night. Its favourite food is crab, which is taken without a great deal of effort. This common octopus frequently moves about by crawling over the sea bottom. Such crawling behaviour as seen in this and other octopods is not found amongst any other living

cephalopods (Lane, 1960). The higher energy expenditure required periodically for crawling and for active hunting might call for a higher oxygen-carrying capacity in the blood of octopods than in that of cuttlefish, which might in turn explain why the data cited earlier show a total blood protein value for *Eledone* intermediate between those for *Sepia* and *Loligo*.

The results of the total protein study, inconclusive as they may be, add considerably to the significance of the electrophoresis results, which would otherwise be mere percentages. In addition, some support is given to the theory regarding the relationship between level of activity and plasma protein content in the cephalopods.

The results of the immuno-diffusion studies, presented in Figure 12, clearly indicate that each case of antigen-antibody interaction has resulted in the production of just a single zone of precipitate. These records are typical of those obtained throughout the study. Each time either a single *Illex* plasma sample or two different samples were permitted to diffuse toward the anti-*Illex* rabbit antiserum, the result was the same, a single precipitin line or two lines which fused in the case of two samples. The use of various combinations of pairs of *Illex* plasma samples has shown that the antigen-antibody complex thus formed is the same in all cases. For example, the complex formed between the *Illex* plasma SB-88 and the antiserum A in Figure 12.C must be the same as that in Figure 12.A. The continuity of the precipitin zone in Figure 12.C indicates that the same complex has been formed between *Illex* plasma SB-302 and the antiserum.

Figures 12.D and 12.E show that the complex formed between *Illex* plasma SB-402 and the antiserum was once again the same.

The implication of these results is that the antigens are in all cases identical in their immunogenic properties, or more simply, that there is only one antigen present, being the same in every sample of *Illex* plasma which has been tested. However, it would be more correct to state that the blood plasma of *Illex* contains at least one antigenic component, which is the same regardless of the sex of the specimen over the period during which the collections were made. It is necessary to include the phrase "at least" because there may be another substance (or substances) present in the plasma, having potential immunogenic properties, which for one or more reasons has failed to elicit the production of antibody in the rabbit blood serum in this study. As stated earlier, the formation of antibody depends upon the immunogenicity (antigenicity) of the injected substance and the responsiveness of the recipient. This responsiveness in turn depends chiefly on the ability of the recipient to degrade and metabolize the injected antigen (Haurowitz, 1968). Thus a recipient is capable of being responsive to one antigen and unresponsive to another.

The nature of the antigenic component of the *Illex* blood plasma, whose presence is demonstrated in this study, remains to be determined. It is not unreasonable to assume that the antigen is the proteinaceous part of the respiratory pigment, hemocyanin, in view of the fact that the presence of this protein in the blood plasma of squids has been well established, and that the results of the electrophoretic study have

suggested that this conjugated protein may be the only protein present in the plasma.

Nothing has appeared in the results of the electrophoretic and immuno-diffusion analyses to indicate any variations due to sexual differences. This may be due in part to the shortness of the period of availability of the squid. No fully mature female specimen of *Illex* has ever been reported in the inshore waters of Newfoundland (Aldrich, 1964; Mercer, 1965). This fact has recently been substantiated by Lu (1968). Generally speaking, the gonads in both sexes were not well developed and no mature or even nearly mature females were found. Among samples taken by Lu in September, 1966 and in October of 1965 and 1966, fewer than 10 of the males were sexually mature. Both Squires (1957) and Lu (1968) state that male squid approach maturity much earlier than females in Newfoundland waters, although Squires (1957) did report three mature females from the waters over the Grand Banks. Since little sexual development apparently occurs or is demonstrable while the *Illex* in the coastal waters, it is not surprising that these studies could not demonstrate changes in the blood chemistry which one might be enabled to attribute to sexual maturation. For such a study, squid of all sizes and stages of maturity would have to be available for analysis. As yet, this great range of sizes and stages is just not available for study.

The true test of any programme of research is, of course, the work accomplished, in terms of the results obtained and their significance, both in themselves and with respect to previous work in the same field. Also to be considered is what further research, if any, is suggested by these results.

This research project has included the first analyses of the proteins of the plasma of the blood of *Illex illecebrosus illecebrosus*, electrophoretic analyses which have yielded promising results. Further studies of these proteins should be undertaken, with a view to determination of the precise nature of the two protein fractions revealed by these analyses. Also important to a thorough understanding of the composition of the plasma are examinations of the ionic composition and the carbohydrate and lipid contents of the blood fluid. With a knowledge of the fluid medium established, a study of the formed elements of the blood should be carried out, which would complete the picture of the blood. This information would seem to be basic to further physiological studies that would elucidate some of the life processes of the animal.

The immuno-diffusion tests described herein are the first such tests performed upon the blood plasma of a teuthoid cephalopod. The results indicate the feasibility of the application of this technique to studies involving blood. The same method should now be tried with other animals as recipients of injected *Illex* blood plasma; in this way additional antigenic components may be revealed, thus forming a broader basis for a systematic serological study involving all three subspecies of the Genus *Illex*. The results of such a study might serve to strengthen the current views regarding the taxonomic relationships within the genus. Indeed, such serological procedures are now indicated as a possible means of interpreting the systematics of cephalopods on inter-familial and inter-generic levels.

CONCLUSIONS

1. A squid may be easily bled. The most plentiful and convenient sources of blood are the posterior venae cavae.

2. The quantity of incidental blood collectable from the posterior venae cavae of a squid is dependent, in large part, upon the degree of distension of surrounding viscera, due to food material present or to the great enlargement of the spermary and accessory organs in mature males late in the season.

3. Upon collection, the blood of *Illex illecebrosus illecebrosus* does not clot, so it is not necessary to follow preventive procedures usually associated with such practice.

4. The electrophoretic pattern of the plasma portion of the blood of *I. i. illecebrosus* indicates a separation of the plasma proteins into two components.

5. The first, or more mobile of these two protein components has electrophoretic mobility which is approximately the same as that of the α-globulin fraction of normal human serum. This protein is believed to be a part of the respiratory pigment, hemocyanin.

6. The second protein fraction has an electrophoretic mobility which is slightly greater than that of human β-globulin. It is quite likely that this protein is also part of the hemocyanin complex.

7. Although few data were acquired, the results of the analysis of total protein content of the plasma indicate a value similar to that quoted for another pelagic (but non-oceanic) species, *Loligo pealei*, but considerably higher than those for less active sepioids and octopods. It may be that total plasma protein is correlated with mode of life and activity. This is especially possible since all other data indicate that the sole protein present is some form of the hemocyanin complex.

8. The blood plasma of *I. i. illecebrosus* contains at least one antigenic component which is capable of eliciting the production of an antibody in rabbit serum. This antigenic component is also believed to be hemocyanin.

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ADDENDA

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2. It is realized that refractometer measurements are only approximations, unless nitrogen or total protein values are actually determined by standard biochemical procedures and plotted against refractive indices. The standard that was used, was not squid plasma; therefore, refractometer measurements are only relative measurements.