AN INVESTIGATION OF THE NORMAL VALUES, INHERENT VARIABILITY AND THERMALLY INDUCED ALTERATIONS IN THE PLASMA PROTEINS OF THE GOLDFISH, *Carassius auratus*.

A Thesis

Presented to

the Faculty of Graduate Studies

The University of Manitoba

In Partial Fulfilment

of the Requirements for the Degree

Master of Science

---

by

James Clarke Fenwick

B.Sc. University of Manitoba, 1962

July 1964

ABSTRACT

The present investigation was undertaken with a dual purpose in mind. First, to determine the normal range of total protein content, and the variability in the electrophoretic patterns of the goldfish, Carassius auratus, and secondly, to investigate thermally induced alterations in the plasma proteins.

Two hundred goldfish were divided into equal lots and acclimated to 5°, 12°, 20°, and 30°C for a minimum of twenty-six days. Plasma samples were taken and total protein determinations made. Fractional separation was carried out using the vertical starch-gel procedure. Relative and absolute concentrations of each of the electrophoretic fractions were determined at the four test temperatures. Each fraction was characterized by a mean mobility and mean size and several fractions related to their human counterparts.

Although goldfish plasma proteins did not fit in with the evolutionary patterns postulated with respect to the complexity of protein fractions, they did concur in protein concentration and the nature of the fractions present. That is, fractions with the mobility of human gamma-globulins were absent, albumin was found only a minor constituent, and lipoproteins formed by far the largest portion of the plasma proteins.

Variations in the abundancies of individual fractions as well as total protein were considerable within each group

as well as between groups. In general, total protein and globulin decreased as the temperature increased, while albumin and the complexity of the patterns increased. The significance of the normal levels, and the thermally induced alterations, were discussed with regard to physiological significance.

ACKNOWLEDGEMENTS

Dr. A. H. Houston of the Department of Zoology deserves my sincere gratitude for his advice, encouragement, and supervision, and also for his assistance in obtaining funds for the present investigation.

TABLE OF CONTENTS

TABLE OF CONTENTS CONTINUED

TABLE OF CONTENTS CONTINUED

LIST OF FIGURES

LIST OF FIGURES CONTINUED

LIST OF FIGURES CONTINUED

LIST OF TABLES

LIST OF APPENDIX TABLES

INTRODUCTION

The present investigation was undertaken with the aim of examining several aspects of plasma proteins of the goldfish, Carassius auratus.

(1) The establishment of normal values for total plasma protein, and following electrophoretic separation, estimation of the normal relative and absolute amounts of the resolved protein fractions.

(2) The determination of thermally induced alterations in the plasma protein values.

(3) To investigate the possibility of utilizing electrophoretic separation of plasma proteins as an index of physiological state with regard to thermal environment.

Despite the copious literature available concerning the thermal relations of fishes (Brett, 1941-52; Fry, Brett, and Clawson, 1942; Fry, Hart, and Walker, 1946; Fry, 1947; Hart, 1947-52; Black, 1953; McCauley, 1958; Kanungo and Prosser, 1959; Hoar, 1962), and the phylogenetic significance of their plasma and serum protein patterns (Lepkoysky, 1929; Drilhon, 1954a; Drilhon, Fine, and Daulous, 1958; Drilhon, 1959; Sulya, Box, and Gunter, 1960; Buecker, 1961), few workers have examined the effect of temperature on the blood proteins (Saito, 1957c; Sorvachev, 1957; Meisner and Hickman, 1962).

Studies on environmental control of protein metabolism have in the past been limited, and have sometimes involved

taken from each of the test groups mentioned above. The values obtained are discussed with regard to those previously reported for this taxonomic group.

LITERATURE REVIEW

Literature pertinant to this topic will be discussed under the following headings:

1. Characterization of the plasma proteins
   a. Albumin
   b. $Alpha_1$-globulins
   c. $Alpha_2$-globulins
   d. Beta-globulins
   e. Gamma-globulins
2. Functions of the plasma proteins
   a. Viscosity effects
   b. Reserve of body proteins
   c. Hydrostatic and osmotic relationships
   d. Transport mechanisms
   e. Blood coagulation
   f. Circulating antibodies
   g. Buffering capacity of the plasma proteins
3. Vertebrate Plasma proteins with particular reference to those of the fishes
   a. The plasma proteins of the higher vertebrates
   b. The plasma proteins of fishes
4. Alterations in the plasma proteins

1. Characterization of the Plasma Proteins

The separation, characterization, and identification of

the plasma proteins preceeded knowledge of their function. As early as 1830 Liebig and Mulder[1] isolated and analyzed blood albumin and fibrin. Since that time the blood plasma and serum proteins have been studied by a wide variety of methods, and have been characterized in terms of a number of physical and chemical properties. Due to the diversity of the methods employed, a profusion of terms has appeared in the literature, and it is now difficult to equate a protein separated by one technique, with that separated by another (Crook et al, 1954; Drilhon, 1960; Amin et al, 1962; Smithies, 1962).

The classical separation of the serum proteins into albumin and globulin fractions was dependent upon the insolubility of globulin in dilute, slightly acidified serum. This first precipitation technique has been replaced by various salt fractionation methods, and these, although providing further fractionation of the globulins, have proven cumbersome. Since each new salt introduced as a precipitating agent fractionated the proteins differentially, it became necessary to redefine the fractions with regard to solubilities at different salt concentrations. With the introduction of the Svedberg analytical ultracentrifuge, the Tiselius moving boundary electrophoresis, characterization and separation of the plasma proteins were facilitated. These techniques, however, introduced new problems into the classification of the blood proteins.

---

[1]Liebig and Mulder - cited in The Plasma Proteins, Vol. I. 1960, p.2. F.W. Putnam, Ed.

Since the proteins were now separated on the basis of different physical properties such as sedimentation constants, and electrophoretic mobilities in a variety of buffers, it again became necessary to redefine the various fractions in terms of these properties.

The proteins of blood serum, as originally separated electrophoretically by Tiselius (1937), were termed albumin, alpha-, beta- and gamma-globulin in order of decreasing anodal mobility. This classification has been largely retained although the fractions obtained by different electrophoretic techniques have been shown to consist of mixtures of many proteins with similar properties (Poulik and Smithies, 1958; Parker and Bearn, 1962; Vandenheuval, 1962). Recently an attempt has been made by Smithies and Poulik (1956) and Poulik and Smithies (1958) to correlate the fractions obtained by paper electrophoresis with those obtained by starch-gel electrophoresis through the use of two-dimensional electrophoresis. This involves electrophoresis on filter paper, followed by a second electrophoresis at right angles in starch-gel. This system furnishes information on the separation of the original fractions on starch-gel. By this technique more than twenty seperate fractions have been resolved in normal human serum (Poulik and Smithies, 1958; Smithies, 1959b). More recently, Hemmings and Jones (1963) combined high-resolution Porath column electrophoresis with starch-gel electrophoresis, and resolved normal human serum into twenty-five distinct fractions.

Although each of the original plasma protein fractions of Tiselius (1937) have been shown to be heterogenous, each has retained a certain independent importance in biological reactions (Madden and Whipple, 1940). For this reason, the name of any subfraction should indicate the original fraction from which it came.. Such a system of nomenclature has been suggested by Smithies (1955); Poulik and Smithies (1958); and Graber (1963). However, these systems are somewhat complicated, and should be replaced by a simpler system which takes into account the physiological role of each fraction as well as its physicochemical properties (Graber, 1963).

For purposes of simplicity the nomenclature used for fractions separated by paper electrophoresis will be used, (Neuhaus et al, 1961; Amin et al, 1962). This system employs the terms albumin, alpha$_1$- globulin, alpha$_2$-globulin, beta-globulin, and gamma-globulin in order of decreasing mobility (See Fig.1). Additional fractions are variously indicated by the addition of Arabic subscripts indicating the mobility of the subfractions relative to other subfractions obtained from the initial fraction. That is, if a third alpha-globulin were found to migrate behind the alpha$_2$-globulin fraction, the new alpha-globulin would be given the name of alpha$_3$-globulin.

As previously stated, a variety of plasma protein fractions have been isolated by two-dimensional electrophoresis

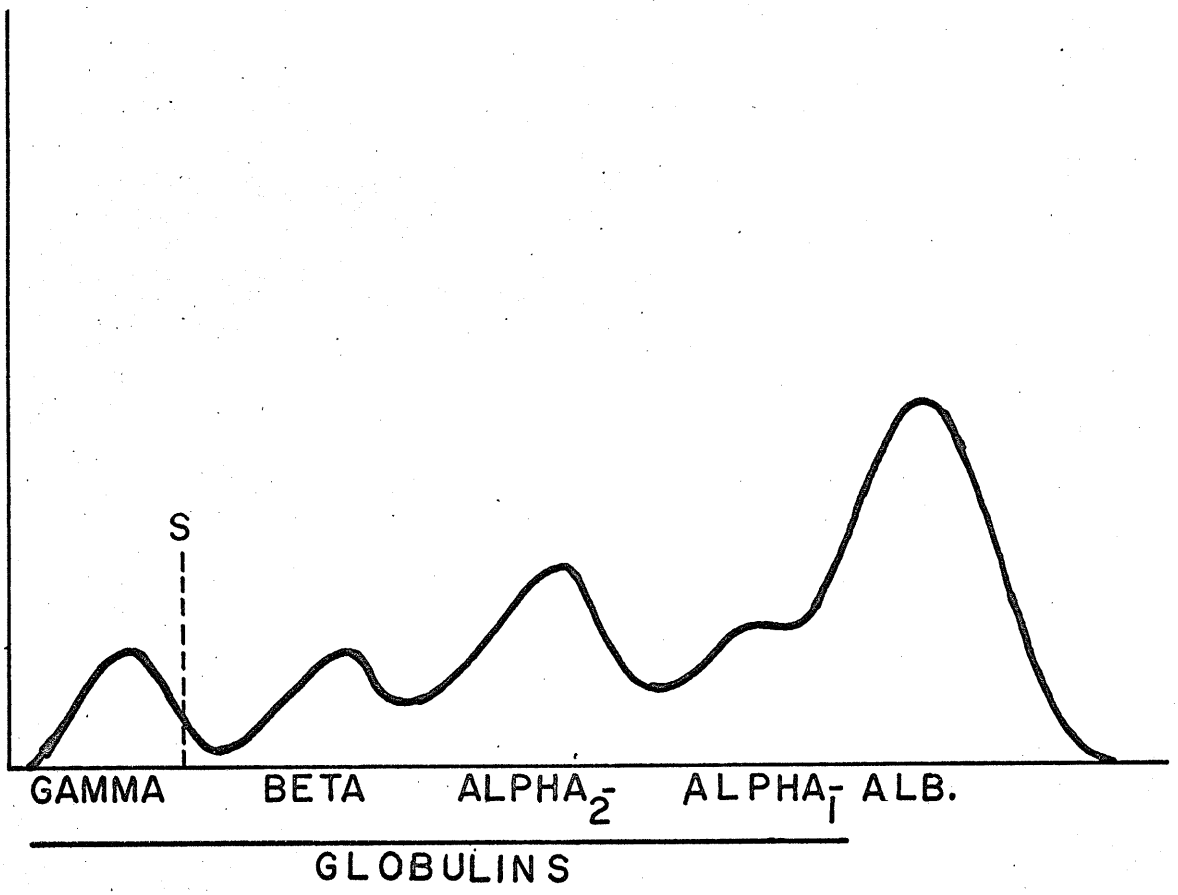

Figure 1. Typical electrophoretic pattern obtained from normal human plasma by paper electrophoresis.

(see Fig.2), and although some have certain physical characteristics in common, they all have some feature specific to themselves. Furthermore, since no single method may delineate all of the plasma proteins, it is necessary to think in terms of groups of proteins when describing the molecular parameters of the plasma proteins (Phelps and Putnam, 1960). Thus, in the ensuing discussion on the characterization of the plasma proteins the various subfractions will be grouped under the older and broader headings, albumin, alpha-, beta-, and gamma-globulins.

a. Albumin

Albumin has the lowest molecular weight of all the plasma proteins. Harper (1957) and Phelps and Putnam (1960), report the molecular weight of human albumin to lie between 60,000 and 70,000, with the most frequently cited value being 69,000. Furthermore, with the exception of an occasionally characterized pre-albumin (Smithies, 1959b), albumin has the highest net charge of the protein components of the plasma at physiological pH (Phelps and Putnam, 1960). Because of these features albumin has most often been found to have the greatest electrophoretic mobility in moving boundary (Tiselius, 1937), paper (Bier, 1959), and starch-gel electrophoresis (Smithies, 1955).

b. Alpha$_1$-globulins

Although migrating on paper in one or two poorly

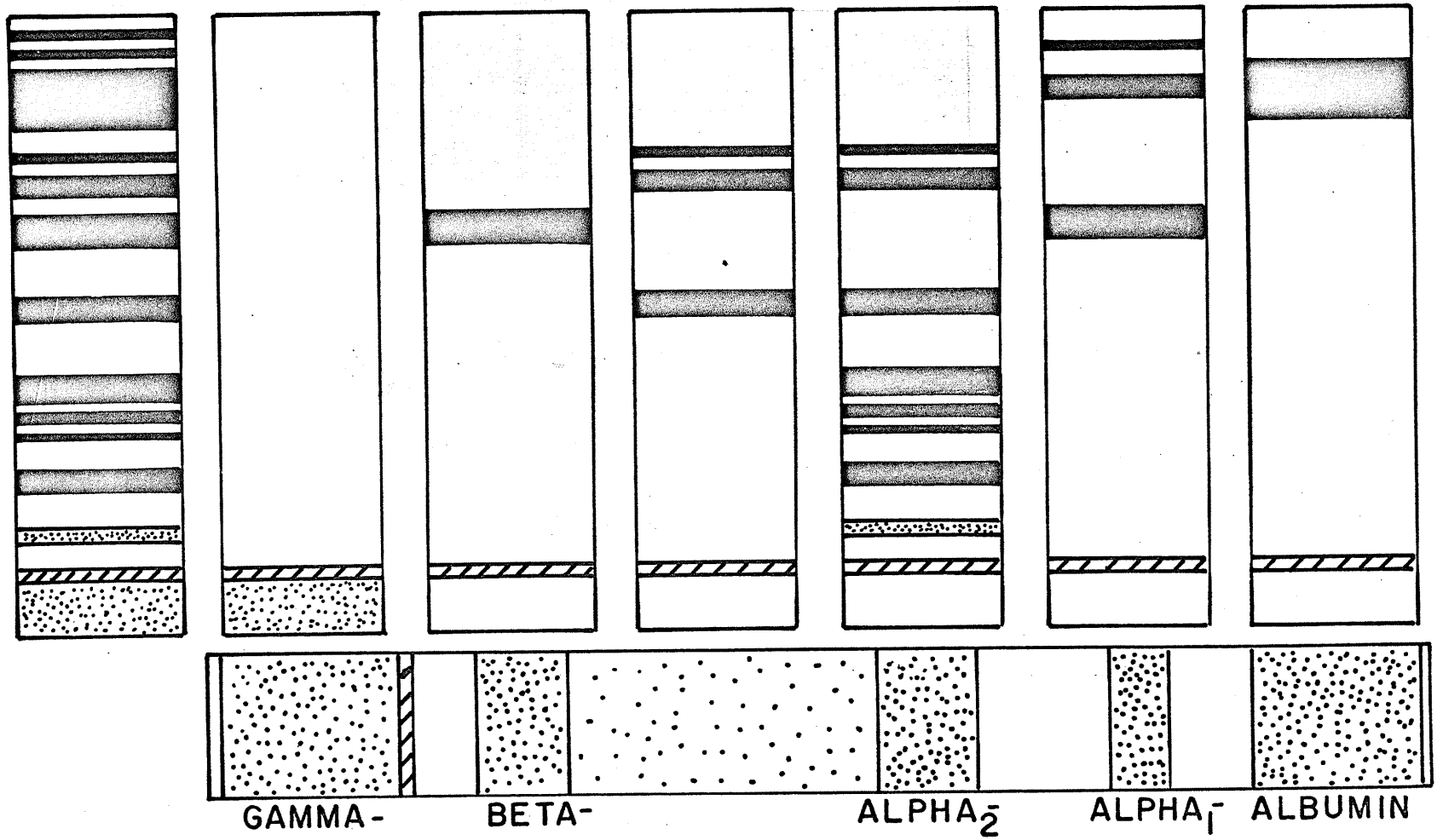

Figure 2. A comparison in diagrammatic form of the zones of human plasma proteins separated by filter paper electrophoresis and one dimensional starch-gel electrophoresis with the zones separated by combined filter paper and starch-gel electrophoresis.

resolved zones, the alpha$_1$-globulins are actually a mixture of proteins with marked physico-chemical differences. In two-dimensional electrophoresis of normal human serum, this band is resolved into three separate fractions. The fastest migrating fraction, which moves with a mobility greater than that of albumin, was originally termed pre-albumin by Smithies (1955), but has since been identified as an acidic alpha$_1$-glycoprotein or synonomously, orosomucoid (Smithies, 1958; Phelps and Putnam, 1960). The other two fractions have not as yet been identified, although Poulik and Smithies (1958) found that in one-dimensional starch-gels, the largest portion of the alpha$_1$-globulin is not resolved from the albumin fraction, while the second, which is probably an alpha$_1$-lipoprotein, forms the first post-albumin of Smithies (1955; Cooper, 1960). The molecular weights of the various proteins in this fraction range from approximately 41,000 for the orosomucoid to between 150,000 and 400,000 for the alpha$_1$-lipoprotein (Dittmer, 1961).

c. Alpha$_2$-globulin

Alpha$_2$-globulin, like the alpha$_1$-globulin fraction, has been found to be a heterogenous mixture (Poulik and Smithies, 1958; Smithies, 1959b; Phelps and Putnam, 1960) which may be separated into at least ten fractions by two-dimensional electrophoresis. Among these fractions are the post-albumins of Smithies (1955), six hemoglobin binding proteins or hapto-

globins, and a large heat-labile glycoprotein, alpha$_2$-glycoprotein (Poulik and Smithies, 1958). While most of the sub-fractions of the alpha$_2$-globulin group are of high molecular weight, considerable variation is evident (See Fig.2). About two-thirds of this fraction is made up of a high molecular weight alpha$_2$-macroglobulin whose molecular weight approximates 820,000 (Phelps and Putnam, 1960). Despite its heterogeneity this fraction is unresolved by paper electrophoresis and moves as a single band between the alpha$_1$- and beta-globulin fractions.

d. Beta-globulins

None of the proteins of this heterogenous mixture (Smithies, 1959b) have been adequately characterized by physico-chemical means (Phelps and Putnam, 1960). Thus far, four fractions have been resolved by two-dimensional electrophoresis, of which one has been identified as a high molecular weight beta-lipoprotein. The beta-lipoproteins have a ratio of fat to protein of approximately 4 : 1 (Harper, 1957), and consequently, while they have a relatively low specific gravity (Poulik and Smithies, 1958) they have a molecular weight which is often in excess of one million (Phelps and Putnam, 1960).

e. Gamma-globulins

This plasma protein fraction is a multifarious group contributing the bulk of the circulating antibodies (Harper,

1957). It normally migrates in gels as a wide band possessing a continuous range of mobilities (Poulik and Smithies, 1958; Smithies, 1959a), but has been shown by more subtle immunochemical means, to consist of more than twenty distinct antibodies in normal human serum (Porter, 1960). According to Phelps and Putnam (1960), "The gamma-globulins appear to consist of a Gaussian envelope of protein components differing continuously in physical and chemical properties, and overlapping in biological function". The most characteristic parameter of the gamma-globulins, in electrophoretic studies, has been their cathodal mobility. Under most electrophoretic conditions they behave as anions while the remainder of the plasma proteins behave as cations (Tiselius, 1937). However, various authors (Poulik and Smithies, 1958; Smithies, 1959ab) have recently demonstrated that some gamma-globulins may show anodal mobility, and Poulik (1957) had earlier demonstrated that the direction of migration of human gamma-globulins could be reversed by changing the buffer system used. Recently, Binette and Schmid (1961) demonstrated various fractions in pathological human gamma-globulin and thus added more proof to the heterogeneity theory of gamma-globulin.

2. Functions of the Plasma Proteins (See Fig.3)

The plasma proteins are a very complex mixture including simple proteins like albumin, and mixed proteins such as the

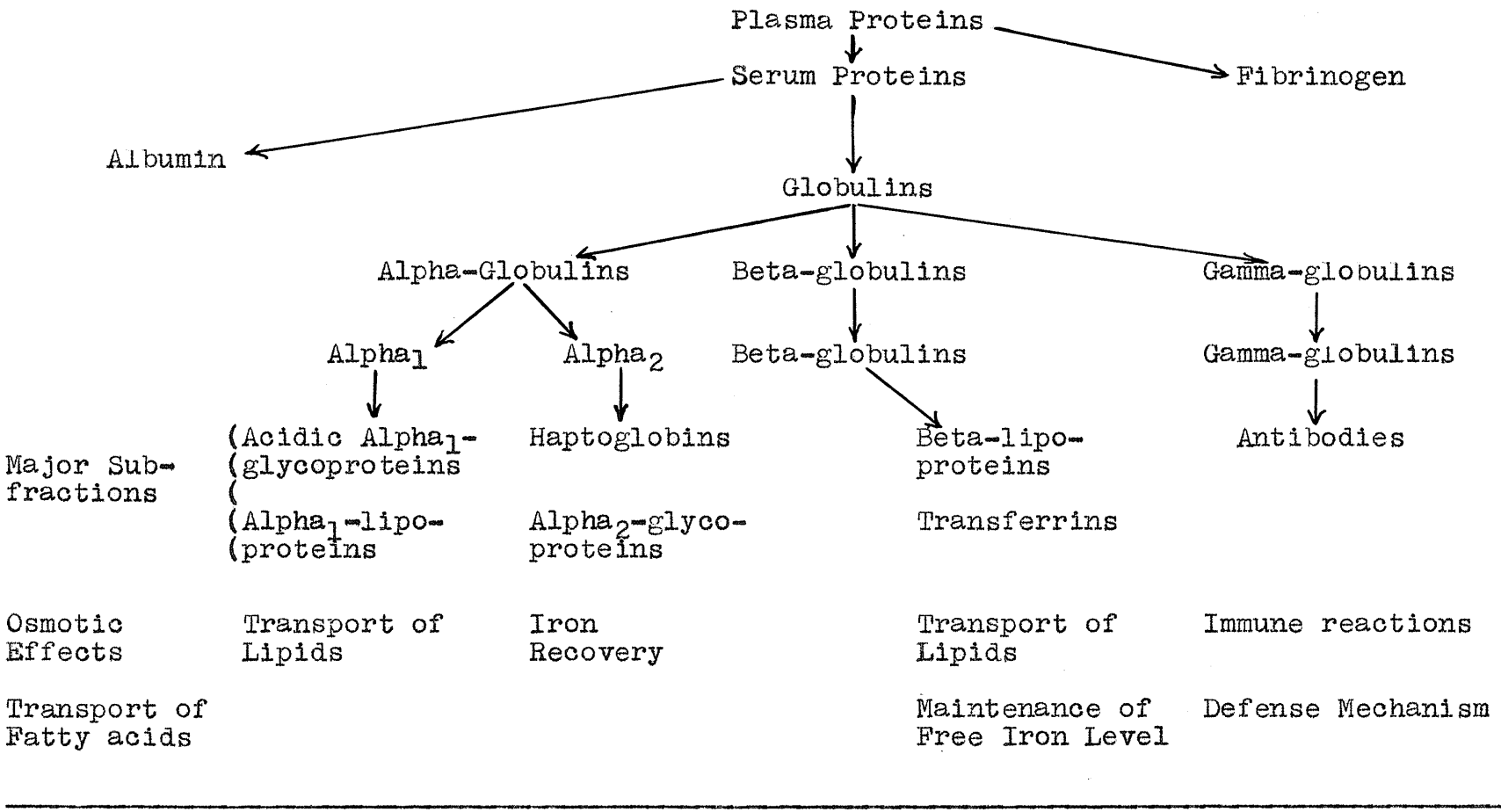

Figure 3

Subdivisions of the Various Electrophoretic Fractions of Tiselius Indicating the Main Function of Each

glycoproteins and lipoproteins. Following the general pattern of West and Todd (1955), the principal functions of the plasma proteins might be summarized by the following statements:

(a) By contributing to the viscosity of the blood they help provide the vascular resistance to blood flow which is essential for efficient heart action.

(b) They serve as a minor source of reserve protein for the tissues of the body.

(c) By virtue of their osmotic pressure (colloid osmotic pressure or oncotic pressure) they help maintain the osmotic relationship between circulating blood and interstitial fluids.

(d) By the formation of complexes they may render various aqueous insoluble substances soluble, and assist in the transport of metabolically active materials.

(e) They provide many of the components of the hemostatic mechanism and therefore are of great importance in blood coagulation.

(f) The gamma-globulin fraction is the principal site of the circulating antibodies.

(g) Since the blood proteins are amphoteric they aid in the buffering of the blood.

A more detailed discussion of these functions may be found in the following text.

a. Viscosity Effects

If the viscosity of water is arbitrarily set at

one, then the viscosity of plasma will be approximately 1.5, and that of whole blood about 3.0. Therefore, the blood cells are three times as efficient in raising the viscosity of the blood as the blood proteins and for all practical purposes, the plasma proteins are not a significant consideration in hemodynamic studies of homiotherms (Guyton, 1961). However, since viscosity increases about two and one-half times with a decrease in temperature from 37°C to 0°C (Ruch and Fulton, 1960), the viscosity effects of the plasma proteins may be of some importance in poikilothermic animals exposed to cold. Increases in viscosity tend to increase peripheral resistance, producing an increase in blood pressure, or reduction in cardiac output. Under most conditions cardiac output is not affected by slight increases in peripheral resistance, and hence one might expect that the normal response will be one of increased blood pressure (Guyton, 1961).

b. Reserve of Body Protein

The plasma proteins serve as a nitrogen reserve during periods of hypoproteinemia (Madden and Whipple, 1940; Prosser et al, 1950; Harper, 1957), and there is also a give-and-take between the proteins of the plasma and those of the tissues (Schoenheimer et al, 1939; Steinberg et al, 1956). A dog, while fasting, was maintained in nitrogen equilibrium by parenteral administration of plasma proteins by Madden and Whipple (1940), and more recently, Cohn (1945) has described how human patients in coma could be similarily maintained.

Furthermore, it has been found by starvation as well as techniques involving plasmaphaerisis[2] (Madden et al, 1950), that fasting dogs retain the potential to regenerate their blood proteins.

c. Hydrostatic and Osmotic Relationships

The maintenance of the osmotic relationship between the circulating blood and the tissue spaces is largely a function of the plasma proteins (Prosser et al, 1950; West and Todd, 1954; Harper, 1957). Since the osmotic concentration of the electrolytes and non-protein organic solutes in plasma and interstitial fluids are, for all practical purposes, identical (Harper, 1957), the oncotic pressure of the plasma proteins is responsible for control of the equilibrium between water and electrolytes in the blood and interstitial fluids (Cohn, 1945).

Of the total osmotic pressure of human plasma (4940 mm. Hg.) the blood proteins contribute about 25 millimeters. However, the interstitial fluids also contain some protein (Guyton, 1961) and this may produce an oncotic pressure of about 10 millimeters (Prosser et al, 1950). Therefore, the effective oncotic pressure of blood over interstitial fluids is 15 mm. Hg. (25-10). This force tends to draw water from the interstitial fluids back into the blood. On the other

[2]A technique involving the withdrawal of blood from an animal, removal of the blood proteins, and reinjection of the deproteinated blood.

hand, this force is opposed by the hydrostatic pressure of the blood which varies from 30 to 15 mm. Hg. from the arterial to venous sides of capillaries. Furthermore, this hydrostatic pressure is also opposed by an interstitial hydrostatic pressure of approximately 8 mm. Hg. caused by the interstitial fluid currently occupying a portion of the available tissue spaces. Hence, the effective hydrostatic pressure at the arterial end of the capillary is close to 22 mm. Hg (30-8), while that at the venous end is about 7 mm. of Hg. (15-8). Thus, on the capillary side, the hydrostatic pressure exceeds the effective oncotic pressure by 7 mm. of Hg (22-15), and this favors the filtration of materials outward from the capillaries into the tissue spaces. On the venous side, the pressure differential is reversed, and a net pressure of 8 mm. of Hg. (7-15) favors reabsorption of water and dissolved materials (Harper, 1957). Albumin, by virtue of it's relatively low molecular weight and high concentration, is the principal plasma protein involved in maintaining this oncotic pressure (Cohn, 1945; Harper, 1957; Bennett and Frieden, 1962). Whereas one gram of albumin yields an oncotic pressure of 5.54 mm. of Hg. the same amount of globulin produces only 1.43 mm. of pressure, (Harper, 1957). Put in another way, the albumin is responsible for about 75% of the total oncotic pressure of the plasma proteins (Foster, 1960).

d. Transport Mechanisms

Although albumin shows little tendancy to form stable lipoproteins (Harper, 1957), it has been assigned an important role in the transport of fatty acids, and hence is important in the transport of lipids from organ to organ (Haurowitz, 1961).

Lipids are also associated with the $alpha_1$-and beta-globulins, their complexes being called $alpha_1$-, and beta-lipoproteins. Of these, the beta-lipoproteins, which migrate electrophoretically in the region of beta-globulin, are the most important in the transport of lipids (Harper, 1957; Lindgren and Nichols, 1960). The importance of these lipoproteins can be seen in the fact that nearly 1% of the weight of serum is made up of lipids (Lindgren and Nichols, 1960), and it is common knowledge that lipids and lipid-like compounds are important in the metabolism and nutrition of animals.

Aside from their important role in lipid transport, the plasma proteins also form complexes with hemoglobin (Herman, 1961); hemoglobin derivatives such as hematin (Haurowitz, 1961); iron (Allison, 1959; Smithies and Hiller, 1959; Beckman, 1962); and other less important substances including various ions (Laurell, 1960); carbohydrates and hormones (Ingbar, 1958; Antoniades, 1960; Haurowitz, 1961).

The hemoglobin binding proteins, or haptoglobins, have

been characterized as alpha$_2$-globulins (Allison and Rees, 1957) and are glycoproteins (Haurowitz, 1961; Herman, 1961). The haptoglobins appear to be of importance in iron and hemoglobin conservation (Laurell, 1960) although Gitlin and Janeway (1960) have questioned this.

The iron-binding beta-globulins, siderophilins or transferrins, act in the transport of iron (Allison, 1959; Smithies, 1959b; Beckman, 1962), and are important in maintaining a physiologically tolerable level of blood iron (Gitlin and Janeway, 1960). They also serve in the transport of iron from the site of absorption in the alimentary tract, and the site of erythrocyte destruction in the reticulo-endothelial system of the liver, to the location of hemoglobin synthesis in the bone marrow (Barber and Sheeler, 1963).

e. Blood Coagulation

This topic has been adequately covered in numerous biochemistry and physiology texts and will not be recapitulated here (Hawk, Oser, and Summerson, 1947).

f. Circulating Antibodies

The plasma proteins include the circulating antibodies and are thus paramount among the defense mechanisms of the body (Harper, 1957). Although the greater part of these antibodies are located amongst the gamma-globulins (Harper, 1957), they have been found in all of the various electrophoretic fractions (Porter, 1960).

g. Buffering Capacity of the Plasma Proteins

By virtue of their amphoteric nature (Harrow and Mazur, 1957), the plasma and cell proteins act as buffers (Pitts, 1963), and indeed, they form the most abundant buffer of the body (Guyton, 1961). Conventionally, one does not think of the plasma proteins as possessing an important role in acid-base balance. According to Pitts (1963), the plasma proteins account for only 3% of the buffering in respiratory acidosis, 1% in respiratory alkalosis, and 1% in the buffering of strong acids and bases. On the other hand, Guyton (1961) stated that in view of the constant exchange between the vascular, interstitial, and cellular elements, that it was not justifiable to speak in terms of the buffering power of blood alone. Hence, taking into account the buffering of the blood and interstitial fluids, Guyton (1961) assigns the following relative buffering powers to the four most important buffers.

Bicarbonate	1.0
Phosphate	0.3
Plasma Protein and Interstitial fluid Protein	0.8
Hemoglobin	1.5

Thus, the blood proteins, though limited in concentration, are probably quite important as part of the first line of defense against slight pH changes of the blood.

3. Vertebrate Plasma Proteins With Particular Reference To Those of Fish.

Phylogenetic variations of the plasma proteins of vertebrates have been reviewed by Engle and Woods (1960), and Dittmer (1961). In general, such reviews approach this problem by indicating changes in total protein, the complexity of the electrophoretic pattern, the relative abundance of the various protein fractions, and the sources of variation.

Electrophoretic plasma protein patterns have been described in numerous vertebrates including man (Smithies, 1955; 1958; Barr, 1958; Harris et al, 1958; Horsefall and Smithies, 1958; Parker and Bearn, 1961; Laudel et al, 1962); the chimpanzee (Buettner-Janusch, 1961); cattle (Ashton, 1957abc, 1958abc); horses (Ashton, 1958c); sheep (Ashton, 1958a); goats (Ashton, 1958b); pigs (Scopes, 1963); the hippopotamus (Weinren, 1960); goldfinches and canaries (Beckman et al, 1962); reptiles (Dessauer and Fox, 1956; Barber and Sheeler, 1961, 1963); amphibians (Dessauer and Fox, 1956; Fox et al,1961); and fish (Cushing, 1952; Hildman, 1956; Sinderman and Mairs, 1959; Sulya et al, 1960-61; Mairs and Sinderman, 1962; Thomas and McCrimmon, 1964). Although all of the previously mentioned workers reported variations in pattern among the individuals of any given species, there are reproducible species-characteristic features in most protein patterns (Deutsch and Goodloe, 1945; Moore, 1945; Latner and Zaki, 1957,

Gunter et al, 1961). Distinct differences have also been seen between the lower and higher vertebrates (Deutsch and McShan, 1949; Gleason and Friedberg, 1953; Dessauer and Fox, 1956; Zweig and Crenshaw, 1957; Cohen and Stickler, 1958; Baril et al, 1961; Gunter et al, 1961; Leon and Wilson, 1961; Dessauer, Fox, and Hartwig, 1962; Fried, 1963). Since these variations have been shown to be genetically based (Smithies, 1955; Ashton, 1957c, 1958b; Smithies, 1958; Beckman et al, 1962) various authors have attempted to demonstrate phylogenetic relationships, and their physiological significance (Irisawa and Irisawa, 1954; Robertson, 1954; Frieden et al, 1957; Fujiya, 1961; Gunter et al, 1961; Leone and Wilson, 1961; Dessaur et al, 1962). However, though definite evolutionary variations in the protein patterns have been reported (Turner, 1937; Deutsch and Goodloe, 1945; Moore, 1945; Deutsch and McShan, 1949; Irisawa and Irisawa, 1954; Robertson, 1954; Dessauer and Fox, 1956, 1958; Gunter et al, 1961; Dessauer et al, 1962), little success has met attempts to relate these changes to physiological function (Irisawa and Irisawa, 1954; Frieden et al, 1957; Leone and Wilson, 1961; Barber and Sheeler, 1963).

Keeping in mind that many exceptions exist, the general pattern in the serum proteins as one ascends the phylogenetic scale may be summarized as follows:

i. The total protein concentration increases.

ii. There is an increase in the amount of albumin

relative to globulin, that is, the albumin/globulin (A/G) ratio increases.

iii. The electrophoretic patterns tend to become more complex, and show an increase in the number of demonstrable fractions.

iv. There is a qualitative and quantitative increase in high molecular weight fractions which exhibit electrophoretic mobilities in the range of human gamma-globulins.

A more detailed discussion of these trends will be found in the following text.

a. The Plasma Proteins of the Higher Vertebrates

The more advanced vertebrates have a higher concentration of blood proteins (Prosser et al, 1950), and generally show an elevated albumin to globulin ratio relative to that of the lower vertebrates (See Table I). It is interesting at this point to refer back to the function of the plasma proteins. As we ascend the phylogenetic scale the hydrostatic pressure tends to increase (Prosser et al, 1950), and thus, the increase in total protein may serve to maintain the balance between the hydrostatic and oncotic pressure as an animal becomes more complex (Rodbard and Feldman, 1946).

While electrophoretic analysis of bird and mammal protein patterns characteristically reveals albumin as the major

TABLE I. Total Plasma Protein and Albumin to Globulin Ratios at Various Levels of Vertebrate Phyla

Group and Species	Reference	A/G	Total Protein in gms.%
Mammals			
Homo sapiens	McFarlane, et al, 1961	2.1	6.5 - 7.0
Hippopotamus amphibius (Hippopotamus)	Weinbraun, 1960	0.52	9.3
Erinaceus europaeus (Hedgehog)	Suomalainen et al, 1961	0.72	7.9
Swine	Miller, et al, 1961	2.0	
Birds			
Chicken (Sp.not given)	Amin, et al, 1962	2.1	
Pidgeon (Sp.not given)	Moore, 1945	2.2	
Duck (Sp.not given)	Deutsch and Goodloe, 1945	2.0	
Turkey (Sp.not given)	" "	2.0	
Reptiles			
Alligator mississipiensis (Alligator)	Baril, et al, 1961	0.1	
Natrix natrix (Grass snake)	Seniow, 1963	0.2 (Oct.) 0.2 (June)	4.56 4.08
Natrix s. sipedon	Cohen, 1954	0.4	4.65
Chelydra serpentina (Snapping turtle)	" "	0.2	4.00

continued

TABLE I (Continued)

Group and Species	Reference	A/G	Total Protein in gms.%
Reptiles (cont'd)			
Chrysemys elegans	Cohen, 1954	0.2	2.43
Amphibians			
Rana catesbiana (Bull frog)	Freiden, et al, 1953	0.11 (tadpole)	1.01
		0.89 (adult)	2.59
Rana herksheri	Frieden, et al, 1957	no alb.	
(Tadpole)			
(adult)		0.13	
Rana pipiens (Leopard frog)	Gleason and Friedberg 1953	(little alb)	0.26
Ambystoma tigrinum (Tiger sulamander)	Hahn, 1962	no alb.	
Ambystoma maculatum	Gleason, et al, 1953	no alb.	0.9
Fish			
Anguille sp.	Drilhon, 1954a	no alb.	7.6
Scyllium stellare	" 1960	traces	3.5
Squalus sp. (Dogfish)	Lepkoysky, 1929	0.30	4.0
Lampetra fluviatilis (Lamprey)	Lepkoysky, 1929		3.6
Lamniformes (3 species)	Gunter, et al, 1961	no alb.	2.2
Cirrhina mrigula (Indian carp)	Das, 1961	0.8	2.69

continued

TABLE I (Continued)

Group and Species	Reference	A/G	Total Protein in gms.%
Fish (cont'd)			
Labeo rohita (Carp)	Das, 1961	0.8	2.87
Catla catla (Carp)	Das, 1961	0.7	3.11
Leuciscus cephalus	Planas and Gras, 1957	0.5	3.6
Barbus fluviatilus	Planas and Gras, 1957	0.3	3.0
Cyprinus carpio (European carp)	Field, et al, 1943	3.6	4.15
	Deutsch and McShan, 1949		3.2
	Sorvacheve, 1957	0.3	3.9
Salvelinus fontinalis	Field, et al, 1943	2.2	3.46
Corogonus clupeoides (Whitefish)	Robertson, 1954		4.2
Ictalurus lacustris (Catfish)	Lepkoysky, 1929	0.3	4.00
Lepidosteiformes (3)(Gars)	Gunter et al, 1961	no alb.	2.87
Clupeiformes (4)	Gunter et al, 1961	0.02	3.89
Siluroideae (Sharks)	Gunter et al, 1961	0.3	2.94
Mugiliformes	Gunter et al, 1961	0.2	3.78

continued

TABLE I (Continued)

Group and Species	Reference	A/G	Total Protein in gms.%
Perciformes (Perches)	Gunter, et al, 1961	0.08	3.70
Acipenser sturio (Sturgeon)	Magnin, 1960	0.34	3.9
Protochordates			
Ascidacea			
Phallusia mammillata	Robertson, 1954		0.03
Thaliacea			
Salpa maxima (Sala)	Robertson, 1954		0.03

component (Common, McKinley, and Maw, 1953; Smithies, 1955; Engle and Woods, 1960), the reptiles and amphibians typically have sera in which the globulins are the most abundant proteins (Deutsch and McShan, 1949; Gleason and Friedberg, 1953; Dessauer and Fox, 1956; Leone and Wilson, 1961). In accordance with this general trend, the majority of fishes have protein patterns composed largely of the higher molecular weight fractions such as alpha- and beta-globulins (Drilhon, 1953,1959; Sulya, et al, 1960; Gunter, et al, 1961).

While Gleason and Friedberg (1953) found little or no albumin in the turtle, Trionyx gangeticus, Coen (1954) reported it's presence in small amounts in five species of turtles. The later studies of Zweig and Crenshaw (1957) and Cohen and Stickler (1958) have indicated the absence of a fraction with the mobility of human albumin, and these workers have

reported that the fastest moving component found in three species of the genus Pseudemys and in the snapping turtle, Chelydra serpentina, had the same mobility as the alpha1-globulin fraction of human serum. More recently, Leone and Wilson (1961) demonstrated albumin to be a normal, though minor constituent of the plasma proteins in nine species of turtles.

From this evidence it is impossible to make any broad statement about the characteristic presence or absence of albumin in the turtle. However, all of the previously mentioned workers agree in the fact that the turtles, like fishes, have a low albumin content relative to the birds and mammals. These findings are supported by certain physiological and histological features. The parenchymal cells of the liver, which are responsible for the production of albumin in man, (Madden and Whipple, 1940), have been described as cirrhotic-like, and nonfunctional, in the livers of fish, amphibians, and reptiles (Elias and Bengelsdorf, 1951).

There may be a functional significance associated with this low albumin in fishes and reptiles. The concentration of albumin in the sera of turtles must be congruous with the low hydrostatic pressure reported for these animals (Rodbard and Feldman, 1946), otherwise, the oncotic pressure of the sera would always exceed the hydrostatic pressure (Leone and Wilson, 1961) and the animal would be subject to hemodilution.

Electrophoretic patterns of reptiles differ substantially from those of the birds and mammals in another feature. Seniow (1963) found beta-globulins to be the most predominant fraction in the grass snake, Natrix natrix, substantiating the earlier finding of Baril et al, (1961), who had reported alpha$_1$-globulin as the major component in young alligators (Alligator mississipiensis). In both cases albumin was present in relatively small amounts, a situation comparable to that described in turtles.

With regard to plasma protein pattern, amphibians do not present a consistent picture. Albumin has been reported as lacking in the salamanders Ambystoma maculatum (Gleason and Friedberg, 1953); Ambystoma tigrinum mavorticum (Hahn, 1962); and others (Dessauer and Fox, 1956). Gleason and Friedberg (loc.cit.) did, however, report the presence of small amounts of albumin in the frog, Rana pipiens, and mudpuppy, Necturus maculatum. Hahn (loc.cit.) has recently noted that the alpha-globulin in Ambystoma tigrinum mavorticum, resolved by paper electrophoresis, separated into a sharp, concentrated band, similar to human albumin. He therefore suggests that alpha-globulin "may serve physiologically as the albumin of the species". On the other hand, Frieden et al, (1957) reported albumin/globulin ratios of 0.86 and 0.50 in the frogs Rana catesbiana and Rana hecksheri, while Rees et al, (1962) have noted the presence of albumin in the frog Xenopus laevis. In

company with the evidence from amphibian metamorphosis, which will be discussed later, Frieden et al, (1957) and Hahn (1962) have suggested that an increased albumin/globulin ratio is an adaptive feature associated with the development of terrestriality. By osmotic influence, increased albumin would aid in the conservation of body water and plasma volume (Frieden et al, 1957) by increasing water regulation in the kidneys and tissues (Hahn, loc.cit.).

The iron-binding proteins or transferrins are unique in the amount of attention they have received from taxonomists and evolutionists. Since Smithies (1955) first commented upon the genetic variations in human transferrins, numerous workers have viewed these proteins as a means of detecting phylogenetic relationships. Genetically controlled transferrin systems have now been found in a number of other mammals (Allison, 1959; Giblette et al, 1959; Barber and Bearn, 1962), birds (Barber and Sheeler, 1961; Beckman et al, 1962), reptiles (Barber and Sheeler, 1961, 1963; Dessauer et al, 1962), amphibians (Fox et al, 1961; Dessauer et al, 1962), and fishes (Drilhon, et al, 1958; Barber and Sheeler, 1961).

Many species including turtles (Barber and Sheeler, 1961); snakes (Dessauer et al, 1962); the chimpanzee (Buettner-Janusch, 1961); and man (Giblette et al, 1959; Smithies, 1959b; Parker and Bearn, 1962) have been found to contain multiple

fraction transferrin systems. However, the more normal situation is that a single transferrin occurs (Dessauer et al, 1962; Barber and Sheeler, 1963). Although associated with the same general physiological function, iron transport, these proteins may show major physico-chemical differences in the serum of diverse species, and in individuals of the same species (Barber and Sheeler, 1963)., The multifariousness of these proteins has led Dessauer et al, (1962) to suggest that they might be amenable to natural selection. That is, homogeneity of transferrins may be a characteristic of older, established species, while those in a more active phase of evolution, and in which natural selection has not adopted the most suitable transferrin, are characterized by transferrin heterogeneity.

b. The Plasma Proteins of Fishes

Blood protein studies in fish have been limited in number, and do not present a concordant picture. Most studies have been confined to species specificity of plasma proteins, and attempts to relate the electrophoretic patterns of fishes to that of man (See Table II). Unfortunately, various authors (Deutsch and McShan, 1949; Saito, 1957ac) and reviewers (Dittmer, 1961) have labelled the protein fractions by number, and have made no attempt to identify them. For this reason, several excellent studies on the electrophoretic patterns of fishes blood could not be included in Table II.

TABLE II. A Summary of the Proteins in Fish Plasma and Serum

Group or Species	Total P. gms.%	A/G Ratio	Alb.	$Alpha_1$	$Alpha_2$	Beta	Gamma	Others	References
Micropogon undulatus (croaker)	1.68	1.13	.242	-	.509	-	.929	-	1
Sciaenops ocellata (red drum)	6.19		.520	1.548	1.139	1.609	1.362	-	1
Leiostomus xanthurus (spot)	2.24		.381	.269	.560	.470	.560	-	1
Pogonius cromis (black drum)	3.10		-	.248	.775	.930	.589	$.558r_2$	1
Logodon rhomboides (pinfish)	4.42		.553	1.282	1.326	.675	.575	-	1
Archosargus probato-cephalus (sheephead)	3.69		.125	.199	1.070	.582	.406	1.291 2	1
Scomberomorus maculatus (Spanish mackeral)	4.47		.492	.756	1.117	.715	1.386	-	1
Cirrhina mrigula (carp)	2.69		1.20	0.88	0.37	0.15	0.096	-	2
Labeo rohita (carp)	2.87		1.32	0.58	0.40	0.20	0.37	-	2
Catla catla (carp)	3.11		1.32	0.27	0.47	0.81	0.32	-	2

continued

TABLE II CONTINUED

Group or Species	Total P. gms.%	A/G Ratio	Alb.	Alpha$_1$	Alpha$_2$	Beta	Gamma	Others	References
Cyprinus carpio (carp)	3.84		0.84	-	-	0.94	0.37	=1.68	3
Leuciscus cephalus	3.68		1.24	-	-	-	1.05	=1.28	4
Barbus fluviatilis	2.97		0.63	.035	.258	.435	1.61	-	4
Crassius auratus (goldfish)			55.7%	-	-	25.5%	9.1%	=17.0%	5
Lepisosteus productus (spotted gar)	2.76		-	-	-	.773	1.987	-	1
Lepisosteus spatula (Alligator gar)	2.71		-	-	.406	1.382	.921	-	1
Lepisosteus osseus (long-nose gar)	3.13		-	.376	.626	2.003	.125	-	1
Brevoortia patronus (Gulf menhaden)	3.84		0.115	1.574	.883	.806	.422	-	1
Dorosoma petenense (Gulf gizzard shad)	2.21		-	.066	1.039	.398	.221	.486r$_2$	1
Dorosoma cepedianum (Gizzard shad)	3.42		-	.770	1.470	.701	.479	-	1
Elops saurus (Ten pounder)	4.87		.214	1.573	1.1831	1.334	.650	-	1

continued

TABLE II CONTINUED

Group or Species	Total P. gms.%	A/G Ratio	Alb.	$Alpha_1$	$Alpha_2$	Beta	Gamma	Others	References
Galeichthys felis (sea catfish)	2.83		.311	.226	.311	.453	1.528	-	1
Bagre marina (Gaff topsail catfish)	3.04		1.064	-	.547	.638	.790	-	1
Mugil cephalus (Striped mullet)	3.39		.102	.746	.881	.339	1.288	-	1
Mugil curema (silver mullet)	4.16		.998	1.040	1.373	.416	.333	-	1
Oligoplites saurus (leather jacket)	4.87		.339	1.839	1.549	.871	.242	-	1
Caranx hippos (common jack)	4.93		.197	.444	.937	1.676	.960	.986r_2	1
Coryphaena hippurus (dolphin)	2.68		.424	.530	.689	.265	.371	.400r_2	1
Cynoscion nebulosus (speckled squeteague)	4.45		.222	.534	.578	.623	.445	.578 .489 .445 .534	1
Cynoscion arenarius (white squeteague)	1.68			.286	.420	.258	.342	.370	1
Salmo fario (brown trout)		0.10	9%						6

continued

TABLE II CONTINUED

Group or Species	Total P. gms.%	A/G Ratio	Alb.	Alpha$_1$	Alpha$_2$	Beta	Gamma	Others	References
Salmo irideus (rainbow trout)		0.96	49%						6
Oligonus macquariensis (Murray cod)		0.41	29%						6
Salmo gairdneri	4.7	0.94	2.24	1.03	0.61	0.61	0.19	-	7
Acipenser sturio (sturgeon)			18.3%			51.7%	8.7%	=8.7% r-fibrinogen 21.2%	8
Sarda chiliensis (bonito)		1.22	55%						6
Zeus australis (John dory)		0.11	10%						6
Allomycterus jaculiferus (porcupine fish)		0.14	12%						6
Acanthistis serratus (wirrah)		0.11	10%						6
Chrysophrys auratus (snapper)		0.22	18%						6
Bulisles hippocrepis (leather jacket)		0.20	15%						6

continued

TABLE II CONTINUED

Group or Species	Total P. gm.%	A/G Ratio	Alb.	Alpha$_1$	Alpha$_2$	Beta	Gamma	Others	References
Pomatomus pedica (tailor)		1.22	55%						6
Sillago maculata (whiting)		0.92	48%						6
Mugil cephala (mullet)	55%	1.13							6
Conger vulgaris			2.4%	18.6%	24.3%	49.3%		$_2$-5.4%	9
Muraena helena			2%	16%	22%	43.5%		$_2$-16.5%	9
Salmo salar (salmon)			14%			12.2%	7%	$_2$-42.5%	9
Petromyzon marinus dorsatus (lamprey)			0	37%	45%	10%	8%		10
Apriodon isodon (sharpnose shark)	2.03		-	-	-	1.096	.934	-	1
Carcharhinus limbatus (ground shark)	2.57	-	-	-	1.130	1.028	.411	-	1
Sphyrna tiburo (bonnet head shark)	1.95	-	-	-	.955	.292	.273	.429r$_2$	1

1. Gunter et al, 1961
2. Das, 1961.
3. Sorvachev, 1957
4. Planas and Gras, 1956.
5. Saito, 1957a
6. Morris, 1959.
7. Meissner & Hickman, 1962.
8. Magnin, 1958.
9. Drilhon et al, 1958.
10. Rall et al, 1961

The plasma protein content of fishes is low in comparison to that of the higher vertebrates (See Tables I and II), and as previously noted, analbuminemia appears as a primitive characteristic (Gunter et al, 1961). In view of the low hydrostatic pressures reported for various fish (Mott, 1957), these findings are in accord with what one would expect from the previous discussion concerning the low values recorded for the turtles. That is, in fish, as in turtles, a low oncotic pressure is necessitated by the low blood hydrostatic pressure if normal fluid exchange between the tissues and blood is to occur, (Rodbard and Feldman, 1947).

There is much controversy in the literature with regard to quantitative and qualitative aspects of fish albumin. Field et al, (1943) and Deutsch and Goodloe (1945) report the absence of albumin in cyclostomes and elasmobranches. This finding has since been verified by Irisawa and Irisawa (1954) in the skate, Raja Kenojei, and the shark Heterodontos japonicus as well as by Drilhon and Fine (1959) who found an absence of albumin in the sharks, Scyllium catulus and Scyllorhinu canicula. Recently, Gunter et al, (1961) working with three species of sharks, Lamniforms, and three species of gars, Lepidosteiforms, and Sulya et al, (1961) with three other species of sharkes, have found analbuminemia to be a consistent feature of the electrophoretic patterns. However, an interesting situation is seen with regard to petromyzontid cyclo-

stomes. While Rall et al, (1961) reported the absence of albumin in the sea lamprey, Petromyzon marinus dorsatus, Thomas and McCrimmon (1964) have recently reported it to be the major blood protein constituent in immature, male, land-locked sea lamprey, Petromyzon marinus.

Analbuminemia, however, is neither universally found, nor restricted to, the lower fishes. Deutsch and McShan (1949) and Magnin (1958) reported the presence of albumin in the most primitive living teleost, the sturgeon, Acipenser sturio. More recently, Sulya et al, (1960) found seven of nine species of salt water teleosts to be without albumin, and Gunter et al, (1961) discovered two cases of analbuminemia among the clupeoid fishes (Dorosoma cepedianum and D. petenense). Furthermore, Morris (1959) reports low albumin levels in the brown trout, Salmo faric, John dory, Zeus australis, and wirrah, Acanthistus serratus. Although most authors concur with Morris (Drilhon, 1954a,1954b; Drilhon and Fine, 1957; Sorvachev, 1957; Gunter et al, 1961), there have been various reports of high albumin concentrations in the teleost fishes (Saito, 1957a; Das, 1961; Meissner and Hickman; 1964). However, since the values have been obtained by a variety of methods, the differences may be technological.

Fish have also been studied by serological techniques, and serological differences have been reported between tuna, Neothannus macropterus, skipjack, Katsuwonus pelanis (Cushing,

1952); goldfish, Crassius auratus (Hildman, 1956); and various species of trout and salmon (Ridgeway, 1962). Furthermore, Field et al, (1943) and Doolittle and Surgenor (1962) also reported that cyclostomes, elasmobranches, and teleost fishes were found to contain a fibrinogen molecule which could be precipitated by human thrombin.

As a general rule, most workers have found low values for, or a complete absence of fractions with mobilities comparable to those of the human gamma-globulins (Deutsch and Goodloe, 1945; Saito, 1957a; Becker et al, 1958). Deutsch and McShan (1949) reported the absence of gamma-globulins in sixteen species of fresh water teleosts, while Engle et al, (1958) discovered similar findings in twenty species of marine teleosts. Further, Becker et al, (1958) found gamma-globulin present in only one species (Thunnus thynnus) of six studied, and Drilhon (1959) reports this fraction in only two out of fifty species (Thymmus sp. and Barbus fluviatus). No evidence of gamma-globulin has been found in the alewife, Alosa pseudoharengus; American shad, A. sapidissima; blueback herring, A. aestivalis; Atlantic herring, Clupea harengus harengus; and the Atlantic menhaden, Brevoortia tyrannus (Sinderman and Mairs, 1958; Mairs and Sinderman, 1962). However, Sulya et al, (1960) believed gamma-globulin to be a normal constituent of the serum of twenty-six species of fish taken from the Gulf of Mexico. Gamma-globulins have also been detected in ells,

Anguilla sp. (Drilhon 1954), the sea lamprey, Petromyzon marinus dorsatus (Rall et al, 1961), and the land-locked sea lamprey, Petromyzon marinus (Thomas and McCrimmon, 1964), as well as other elasmobranches (Saito, 1957a; Engle et al, 1958; Fujiya, 1961). It appears, therefore, that while gamma-globulin is characteristically present in the lower fishes, the more advanced ones are less consistent with respect to its occurance. However, referring back to the characterization of the gamma-globulins (p.12), it may well be realized that the functional gamma-globulins of some fishes might show anodal mobility. Thus, it is not justifiable to say that fish have no gamma-globulins, but it is quite correct to say that some fish have no electrophoretic fraction with the mobility of human gamma-globulin.

The most characteristic feature of the blood protein pattern of fishes is the predominance of the alpha- and beta-globulins (See Table II), and the correspondingly high lipoprotein levels. Drilhon (1959) and Sulya et al, (1960) have reported alpha- and beta-globulins to be the most predominant fractions in over fifty species of fish. These workers have recently been supported by the findings of Magnin (1960), in the sturgeon, Acipenser sturio, and by Rall et al, (1961) in the lamprey, Petromyzon marinus marinus. Furthermore, Field (1943) Morris (1949), Das (1961) and Sulya et al, (1961) have found lipid and cholesterol levels in the blood of many salt

and fresh water fishes that far exceed those in man. These findings are consistent with the high lipoprotein content reported in many fish (Drilhon, 1958; Sulya et al, 1960; Das, 1961).

It has been shown in a number of species of fish, that, unlike mammalian lipoproteins, the fish lipoproteins often migrate with the albumin band during paper electrophoresis (Drilhon, 1954; Saito, 1958; Morris, 1959). This probably accounts for the high albumin reported in some fish (Saito, 1958; Meisner and Hickman, 1962). That is, where one worker measures the albumin fraction per se, another may differentiate it into two functionally dissimilar proteins, thus reporting a much lower albumin content for the species.

4. Alterations in the Plasma Proteins

A variety of alterations in the plasma and serum proteins of fish have been described in the literature. Turner (1935) reports a decrease with age in the total oncotic pressure of trout blood. On the other hand, Saito (1957d) found an overall increase in two species of marine elasmobranches (Mustelus griseus and Dasyatis akajey). This latter report is substantiated by that of Magnin (1960) who found an increase in both total protein and albumin content with age in the sturgeon, Acipenser sturio. The albumin/globulin ratio in this instance increased from 0.26 in young sturgeon to 0.34 in older animals. Electrophoretic analysis of pre-spawning and

parasitic sea lampreys, Petromyzon marinus, demonstrated a two-fold increase in total protein (Thomas and McCrimmon, 1964).

Variations also occur at the time of metamorphosis in various animals. Since metamorphosis is a period of rapid ontogenetic development, it has received much attention. For obvious reasons the amphibians have received the greatest attention in this respect (Frieden et al, 1957; Herner and Frieden, 1960; Bennett and Frieden, 1962; Hahn, 1962). However, ontogenetic changes have also been described in the chicken (Amin et al, 1962); swine (Miller et al, 1961; rat (Wise et al, 1963); lamb (Silverstein, 1964); man (Das and Bhattacharya, 1961) and others (Moore et al, 1945; Marshall and Deutsch, 1950; Moore, 1959; Engle and Woods, 1960). Studies on changes in the blood proteins of fishes during metamorphosis are limited to the lower forms and few comprehensive examinations have been made (Saito, 1957d; Rall et al, 1961; Thomas and McCrimmon, 1964). The results of such studies have generally been in agreement with those from higher vertebrates in that most authors report an increase in the total protein and in the number of demonstrable fractions.

Sex based differences have been noted by various workers including Drilhon (1954); Magnin (1960); and Thomas and McCrimmon (1964), but the results are fragmentary and do not, as a rule, agree . Urist et al, (1961) induced an increase in total protein in the kelp bass, Paralabrax clathratus, by injection with estrogens but such studies have been seriously

hampered by the individual variations among the test animals.

Drilhon (1954), Sorvachev (1957) and Fujiya (1961) have studied the effects of starvation on plasma proteins, and although they have reported species specificity with regard to these changes (Sorvachev, loc.cit.), all have found an eventual decrease in total protein level. This is largely accountable in terms of a decreased albumin content.

Very few studies have been carried out on the effects of extrinsic factors upon plasma protein pattern, although Sinderman and Mairs (1958) did find decreased serum albumin in sea herrings suffering from a fungus disease, Icthyosporidium hoferi, as well as certain qualitative alterations in the electrophoretic pattern. Fujiya (1961) made a study of the effects of various concentrations of industrial wastes on the plasma proteins of fishes, but very few such studies have been carried out under controlled conditions.

The effects of climatic factors on the blood proteins of fishes have been seldom studied, and what reports there are show different results. Meisner and Hickman (1962) examined the effect of temperature and photoperiod on the plasma proteins of the rainbow trout, Salmo gairdneri, and found an elevated albumin/globulin ratio at 8°C relative to that at 16°C. They reported that this decrease was the result of a decreased albumin content coupled with an increase in beta-globulin at the higher temperature. Their findings disagree with those of Saito (1957c) and Sorvachev (1957). Saito (1957c)

found a decreased total protein and albumin in both the carp, Cyprinus carpio and mackerel, Scomber tapeinocephalus, during the winter months, and Sorvachev (loc.cit.), working with the same species of carp, noted a decrease in albumin as well as in alpha- and beta-globulins during the colder months. However, since the winter corresponds with a decrease in food consumption (Sorvachev, 1957) it was difficult to determine whether these changes were due to the temperature per se, or to the accompanying alterations in feeding habit.

MATERIALS AND METHODS

1. Experimental Animals

Goldfish (*Carassius auratus*) of both sexes and ranging in weight from 5 to 55 grams, with a mean weight of 13.4 ± 1.4 grams, were obtained from the Goldfish Supply Company, Stouffville, Ontario. Although no attempt was made to sex the fish, it was noted that the great majority were in a gravid condition.

The fish were fed a specially prepared fish food, obtained from the Manitoba Fisheries Department, and were allowed to feed *ad libitum* daily.

2. Aquaria and Conditions of Acclimation

The aquaria used were of wood construction and measured 20" x 18" x 38", with a total capacity of 50 Imperial gallons. The tanks were painted with an inert epoxy-resin[1], and were filled to half capacity with distilled water. The tanks were held in constant temperature rooms at air temperatures below those of the tanks. Constant water temperatures were then obtained by use of 300 watt, Teflon-wrapped, copper-coil, heating elements used in conjunction with thermistor thermoregulators[2].

---

[1] Fisher Epoxy-resin Laboratory Paint. Fisher Scientific Company.

[2] Thermistemp Temperature Controller: Model 63, Yellow Springs Instrument Company Ltd., Yellow Springs, Ohio.

The water in the tanks was elutriated by the use of sub-surface, air operated, plastic filters, charged with fine glass wool and activated coconut charcoal.

Oxygen content of the water was maintained near saturation by continuous aereation.

Photoperiod was controlled at sixteen hours per day by means of tight fitting hoods equipped with incandescent 40 watt bulbs[3] placed approximately 15" above the surface of the water and activated by Intermatic Time Switches.[4]

Two hundred fish were divided into four equal lots and were acclimated at 5.0 ± 0.5°C, 12.0 ± 0.5°C, 20.0 ± 0.5°C and 30.0 ± 0.5°C for a period not less than 26 days. This interval exceeds the period suggested by Brett (1946) and Anthony (1961) as required for acclimation to these T's. This range of temperatures also approximates the range of thermal tolerance of the goldfish (Brett, 1956).

Under these conditions the fish appeared to remain in a healthy condition and the mortality rate was low.

3. Method of Plasma Collection

Prior to sampling the fish were anaesthetized in tricaine methanesulphonate (MS 222, 1:10,000), and were considered to be fully narcotized when incapable of righting reactions. The

---

[3]General Electric Lumiline 52: General Electric Company.

[4]Intermatic Time Switch: Model T101, International Register Co., Ch. Ill.

animals were then lightly dried with a paper towel, and the scales cauded of the anus removed by scrapping. A clean sharp scalpel was used to amputate the caudal peduncle, and the blood was allowed to run into polyethylene dishes lightly dusted with dry disodium ethylenediaminetetraacetate (EDTA) to prevent coagulation. Samples were immediately centrifuged at 10,000 rpms. for four minutes, after which the plasma was pipetted off and stored in stoppered centrifuge tubes at -10°C. Samples with more than slight traces of haemolysis were discarded.

There is some controversy regarding the effect of freezing and thawing on electrophoretic patterns Goswami and Barue (1959) found that there was no change in the paper electrophoretic pattern of protein following cold storage. Mathews and Buthala (1956) also reported no changes in the paper electrophoretic pattern of swine serum following repeated freezing and thawing. On the other hand, Pensinger et al, (1959) reported that such treatment cause changes in the relative percentages of several protein fractions, while Smithies (1955) reported variations in the abundance of alpha-2 globulin fractions when the proteins were stored for more than one week. In view of the conflicting reports, care was taken to ensure that all samples were subjected to the same treatment. All samples were stored frozen for a period of at least one week prior to testing, and no sample that had been frozen more than

once was used. By this method it was hoped that variations in the pattern due to the effects of freezing and thawing were held to a minimum. Since the absolute pattern was not as important, in this study, as the variations in pattern due to the different acclimation temperatures, specific effect of freezing and thawing was not determined.

4. Electrophoretic Technique

a. General Discussion

The electrophoretic technique employed in this experiment involved the starch-gel procedure of Smithies (1955, 1959a). This technique is of the zone electrophoretic type, and is commonly called "vertical starch-gel electrophoresis". It was chosen for this experiment because it combines the advantages of zone detection by staining, high resolution, freedom from serious absorption effects, and good reproducability. This improved starch-gel technique has the further advantage that the sample may be introduced initially into the gel as a narrow and even zone.

Following electrophoresis the gels were sliced and stained. Staining with Amido Black was carried out as recommended by Smithies (1955). Since this dye apparently does not obey Beer's Law (Smithies, 1959b; Jansen, 1962; Matsui and Yaeno, 1963), absolute amounts of the various protein fractions may not be accurately determined. However, "the relative amounts, so

determined, are useful in the quantitative estimation of deviations from normality" (Smithies, 1959b).

In order to check electrophoretic reproducibility and the staining technique, a standard reference plasma (Lab-Trol[5]) of known composition was used. Such samples were stained and analysed in the same manner as experimental samples. Four fractions were found and named, in order of decreasing anodal mobility, albumin, globulin-3, globulin-2, and globulin-1. The mean of the area under each fraction, as well as the mean relative percent of each fraction, was determined together with standard deviation, standard error, and 95% fiducal limits of the mean. As can be seen from Table III, the values were distributed in approximately normal fashion, and the value for albumin obtained (65% of total), was close to that as listed for the sample (67%). The albumin:globulin ratio of 2.0 was close to that of the actual A/G ratio of 2.1. It may also be noted that the variations in migration distances of the various fractions were small relative to the total migration of the fractions. This latter finding suggested that a high degree of reproducability might be expected with regards to fraction mobility. Despite this, however, it sometimes proved difficult to separate bands with closely similar mobilities.

Other important sources of error inherent in this technique included; nonuniformity in gel thickness, and deformation

---

[5]Lab Trol: Fisher Scientific Company.

TABLE III. Analysis of Control Serum Showing Inherent Variation in the Technique

Fraction	% of Total Area Under Fraction: Mean with 95% Fiducal Limits (cm)	Standard Deviation in cm.	Standard Error in cm.	Mean Fraction Position: Mean with 95% Fiducal Limits (cm)	Standard Deviation in cm.	Standard Error in cm.
Globulin 1	1.8 ± 0.2	1.0	0.2	6.0 ± 0.2	0.5	0.1
Globulin 2	5.0 ± 0.6	1.9	0.3	6.9 ± 0.2	0.4	0.1
Globulin 3	27.0 ± 2.4	6.9	1.2	8.6 ± 0.2	0.9	0.1
Albumin	66.1 ± 3.1	9.3	1.6	15.5 ± 0.2	0.7	0.1

	Mean Fraction Size: Mean with 95% Fiducal Limits.	Standard Deviation in cm^2	Standard Error in cm^2	Comparison of Values: Value Compared	Known Value	Determined Value
Globulin 1	1.1 ± 0.2	0.6	0.1	A/G Ratio	2.1:1	2.0:1
Globulin 2	2.8 ± 0.4	1.3	0.2	Percent Albumin	67%	65%
Globulin 3	15.6 ± 1.8	5.5	0.9	Percent Globulin	23%	25%
Albumin	38.2 ± 2.3	10.2	1.7			
Total Curve	57.8 ± 4.1	12.5	2.1			

of gels. The first was largely overcome by slicing the gel longitudinally, in the horizontal plane, after placing the gel in a slicing tray of uniform depth. The second was partly overcome by carrying out electrophoresis in a cold room which increased the rigidity of the gel (Matsui and Yaeno, 1963). Furthermore, deformation by stretching, during the handling of the gel, was eliminated by placing the gel on a wet surface prior to densitometric analysis.

b. Dimensions of the Apparatus[6]

A plexiglass tray[6] with internal dimensions of 6 mm in depth, 121 mm in width, and 318 mm in length was used as both the gel mould during preparation, and as support during electrophoresis. This tray was fitted with removable end pieces, and a removable top of flexible polyethylene 1.5 mm thick. The top was fitted with a precut eight slot mould. The individual sample slots were 10 mm in length by 5.5 mm in depth, and 1.0 mm in width. Located between each slot former was a circular saw cut 4 mm wide so that a ridge 1.5 mm in height was formed between each slot. This ridge prevented the sample from one slot contaminating the sample in another. The slicing tray had the same dimensions as the gel tray but was only one half as deep. The reservoirs for the bridge solutions, as well as a specially built platform for the levelling of the gels, were supplied

---

[6]Exact measurements along with construction details may be found in Smithies, 1959a. Biochem. Jour. 71, pp.585-586.

with the gel tray.

c. Composition and Preparation of the Gels

"Starch hydrolyzed for gel electrophoresis" was obtained from the Connaught Medical Research Laboratories and the procedure for the preparation of the gels followed the prescribed instructions[7] and those described by Smithies (1955, 1959a) and Poulik and Smithies (1958). Borate buffers (See Table IV) with a pH of 8.90 ± 0.05 were used throughout these experiments.

TABLE IV. Composition of Starch Gels for the Various Lots of Starch Used.

Lot No.	Gms. of Starch/100 mls of Buffer Used	Composition of the Buffer
177-1	12.4	0.0260 M Borate 0.0104 M NaOH
188-1	11.8	0.0260 M Borate 0.0104 M NaOH
190-1	11.3	0.0270 M Borate 0.0108 M NaOH

The amount of starch used for each 100 ml of buffer varied depending upon the suppliers recommendations (See Table IV) for the particular lot of starch used, the average amount used being 11.8 grams per 100 ml of buffer. Since 500 ml of gel were prepared at a time, the average total amount of starch

[7] Three lots of starch were obtained from the manufacturer each with different recommendations with regards to buffer and starch composition. For details see Table II.

used in each gel amounted to 59 grams.

To prepare the gel, the proper amount of "Starch hydrolyzed" was added to 500 ml of buffer in a 2000 ml vacuum flask. This mixture was then stirred until a homogenous suspension of starch was produced. The flask was then heated over a naked flame with continual swirling to ensure even heating of the suspension. Heating was continued until the suspension of starch was just short of boiling, at which point the starch grains ruptured and a viscous, homogeneous solution of starch was obtained. Further heating and swirling caused the viscosity of the solution to drop well below the maximum seen at the time of rupture of the starch grains. The solution was then degassed by vacuum to remove air bubbles. The degassing time was kept to a minimum in order to prevent excessive loss of buffer, since losses result in increased starch concentration which then effects protein mobility during electrophoresis, (Smithies, 1962). The starch solution was poured into the gel tray, and immediately covered with the polyethylene top, which had previously been coated with a thin layer of mineral oil, and heated to 70°C. Entrapment of air bubbles was largely avoided by lowering the top in a diagonal manner from end to end. Any air bubbles which were trapped could be readily removed by gently pressing out the excess gel with a flat piece of plastic. This latter procedure did not remove the air bubbles trapped in the circular saw cuts between the slots. How-

ever, since air bubbles which were trapped between the slots and the cathode end of the gel did not affect migration, this problem was largely overcome by initially placing the top such that the slot formers were as close to the cathode end as possible. Then, when the slots were moved down to their final position, by sliding the top down the gel, the bubbles which were trapped in the circular saw cuts were left behind in that part of the gel between the slots and the cathode. The position of the slots, with regard to distance from the cathode, was important since the voltage gradient varies over the length of the gel (Smithies, 1955). The distance from the cathode selected for this experiment was 8.0 cm., and was constant in all runs. The gel was then allowed to cool to room temperature and in doing so became semi-transparent, and fairly rigid. The top was removed by carefully prying the sides and the slot formers loose from the gel with a flat spatula.

Once the top had been removed the gel was used immediately as Smithies (1955) had previously demonstrated that gels allowed to stand lost some of their resolving power.

d. Introduction of the Sample

Since all plasma samples had previously been frozen, they were removed from the freezer about 30 minutes before use, and allowed to come to room temperature. With the use of a micro-pipette, 60 microliters of sample were added to each slot. Of the eight slots one was used as a control and was filled with a specimen of "Lab Trol"; the remaining seven being filled

with samples from different fish. In the case of the control, only 40 micro-liters were used as samples of larger size contained too much albumin which, tending to diffuse laterally, obscured neighboring samples.

The ends of the casting tray were removed, and with care being taken not to trap any air bubbles, each slot was covered with a glass cover slip. The glass slips were then covered with molten petroleum jelly (at about 45°C) so that an air-tight and water-proof seal was formed. The cover slips prevented liquid petroleum jelly from seeping into the slots, and the samples, therefore, remained in uniform contact with the gel surface. The top surface of the gel was then covered with a non-porous, transparent film (Saran Wrap[8]) leaving bare the ends with which the filter paper wicks came into contact (See Figure 4).

e. Electrical Connections

Electrical contact with the gel was made with six thicknesses of filter paper[9] which had previously been soaked in borate bridge solution (0.3 M of borate and 0.06 M of NaOH). The arrangement of the apparatus during electrophoresis may be seen in Figure 4. The chambers a, b, and c were filled with 500 ml of borate bridge solution while chamber d was filled with 10% NaCl solution. Chamber A, containing the Ag/AgCl

[8]Saran Wrap: Dow Chemical Company.

[9]Whatman Filter Paper No.1: W. & R. Balston Co.

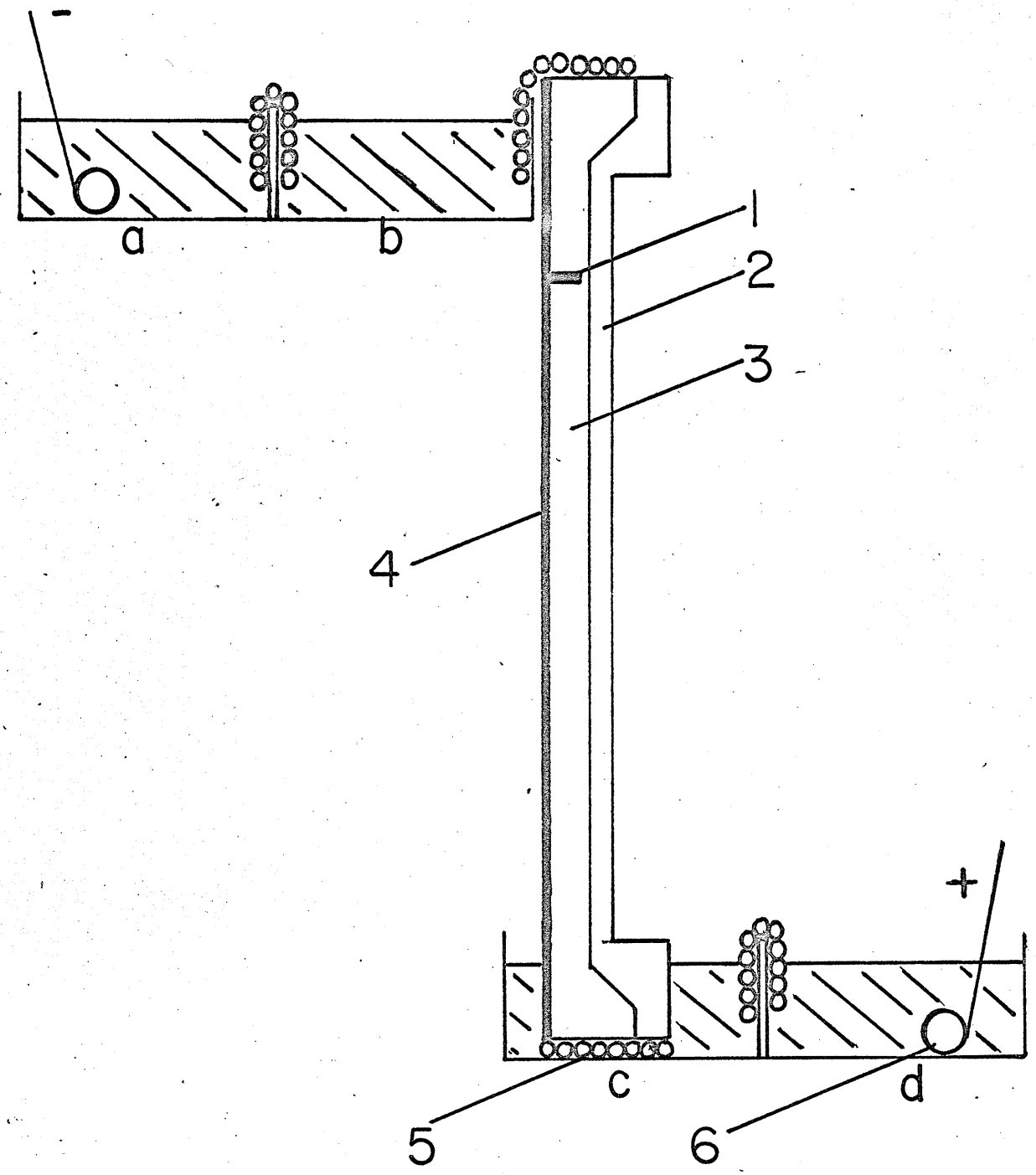

Figure 4. The experimental arrangement for vertical starch-gel electrophoresis (Smithies, 1959a).

a and b = trays containing bridge solution. c = tray containing bridge solution with approximately 12 thicknesses of filter paper at the bottom on which the end of the gel rests. d = tray containing 10% NaCl solution for the positive reversible Ag/AgCl electrode. 1 = position of sample slots. 2 = gel tray. 3 = gel. 4 = water proof seal (Saran wrap). 5 = filter paper. 6 = anode.

cathode, was connected to chamber b by eight thicknesses of filter paper, while chamber c and d were connected similarily. In addition, the bottom of chamber c, which came in contact with the gel, was lined with ten thicknesses of filter paper, while chamber d contained a solution of 10% NaCl for the positive reversible Ag/AgCl electrode. The electrodes[10] were coiled reversible electrodes made from electrolytically purified silver coated with silver chloride. The use of these electrodes was recommended by Smithies (1955) as the electrode process involves only the transport of sodium chloride from the positive to the negative electrode chamber, and no hydrogen or hydroxyl ions are involved (anonymous). Consequently, use of these electrodes prevents the pH changes in the gels which accompany the use of platinum or carbon electrodes.

The electrodes were attached to a regulated power supply[11] which could deliver power at either constant voltage or constant current.

f. Conditions of Electrophoresis

The tray was set up, with the cathode at the top and the anode at the bottom (See Figure 4), so that migration would occur in a vertical, downward direction. Electrophoresis con-

---

[10] Ag/AgCl Electrodes: Otto Hiller Co., Madison, Wisconsin.

[11] Power Supply: Heathkit Regulated Power Supply, Model 1P-32: Daystrom Limited, Cooksville, Ontario.

sisted of subjecting the gel to a constant voltage of 4.4 volts per cm for a period of twenty hours at a constant air temperature of 10°C. Under these conditions the current reading at the beginning of the run was between 14 and 15 milliamperes (mA), and rose to 16 or 17 mA during the course of the run. Although this increase in amperage had no apparent effect on the mobility of the proteins, a marked shrinkage of the gel at the anodal end did accompany any excessive increase in the current. Both the shrinkage and the increased current appeared to be caused by the difference in concentration between the buffer in the gel and the buffer in the bridge solution. It appeared that as the two solutions in chambers c and d came into equilibrium with each other, the osmotic concentration of chamber c increased markedly causing a movement of buffer solution out of the gel and into the anodal chamber. This movement caused the anode end of the gel to shrink, and at the same time caused an increase in the concentration of buffer in the anodal end of the gel, thereby decreasing the resistance of the gel to the current and raising the current flow. When shrinkage became so pronounced as to interfere with protein migration, the gel was discarded, and fresh bridge solutions were prepared.[12]

---

[12]Under normal conditions the same bridge solution was used for a period of one week. The number of electrophoretic runs appeared to have no influence on the length of time that any one bridge solution could be used.

5. Detection of the Proteins

a. Slicing and Staining

Following electrophoresis the gel was carefully removed from the gel-tray by turning it out onto a piece of plastic screening which had been cut to size. The use of this screen during the staining procedure made it possible to reduce deformation of the gel. The gel was then placed in the slicing tray such that the slots were away from the cutting block and the circular saw cuts were on top. Gels were sliced longitudinally in the horizontal plane by means of a sharp, single edged dermatome knife. Sliding of the gel along the slicing path was prevented by drying the surface of the gel which came in contact with the floor of the slicing tray. After the gel was sliced the two halves were separated, and the top half discarded, while the other half was retained for staining.

The gel, still on the screening, was stained in an aqueous solution of 1% Amido black and 5% acetic acid for twelve minutes. The actual staining time, and the concentration of acetic acid, are not critical (Matsui and Yaeno, 1963). Although Fazekas et al, (1963) and Jansen (1962) have reported that Amido black is not a satisfactory dye for the evaluation of protein concentrations on either cellulose acetate or agar-gel, Matsui and Yaeno (1963), and Smithies (1963) have used this dye with reasonable success on starch-gel preparations.

b. Destaining of Stained Gels

After staining, the gel was removed, and excess dye

washed off with water. Destaining was carried out in an automatic gel washing machine[13] (Pert et al, 1959) with the carbon electrodes attached to a 12 volt Schauer Rectifier. The washing machine was filled with an aqueous acetic acid solution (approximately 3%) and the current flowing across the electrodes adjusted to five amperes. Under these conditions the gel could be destained in about thirty minutes and was then nearly clear, except for the stained proteins (See Figure 5). Although prolonged washing reduced the standard deviation of the apparent proportions of the fractions (Matsui and Yaeno, 1963), it also reduced the efficiency of the densitometer in recording fractions with low protein concentrations.

6. Scanning of Stained Electrophoretic Strips

Stained and washed gels were cut longitudinally into eight, separate, electrophoretic strips, each containing the electrophoretic pattern of a particular sample. Each strip was scanned using a Photovolt Model 525 transmission densitometer in combination with a self-balancing potentiometer-recorder[14]. The transmission density unit (Model 52-C) was fitted with, a scanning stage designed to accomodate starch-gel strips, and a motor drive assembly unit, which automati-

[13]Automatic Gel Washing Machine: Otto Hiller Company.

[14]Both the densitometer and the recorder were obtained from the Photovolt Corporation, New York, N.Y.

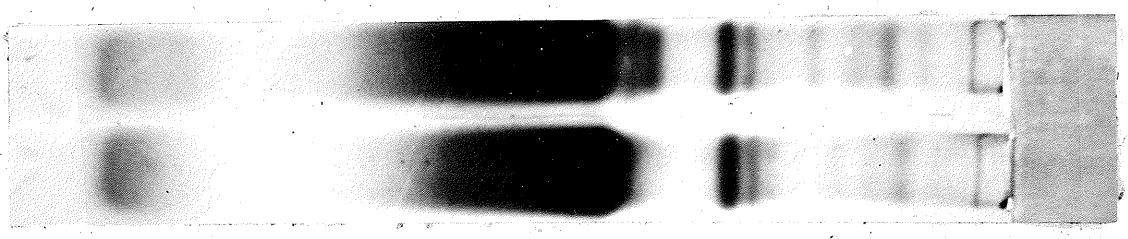

FIGURE 5. Electrophoretic Pattern of Goldfish Plasma Following Staining and Destaining.

cally drove the starch strips past the scanning head at a speed of 42 mm/minute. The chart speed, on the recorder, was 51 mm/min., hence, the electropherogram of the gel was expanded 22% in length. The slit size of the light from the stabilized light source was 1.5 by 0.2 millimeters.

The variable response recorder offered a choice of twelve response functions, ranging from linear (% transmission) through several semi-logarithmic functions, to logarithmic (optical density) and further to trans-logarithmic functions (true concentrations)[15]. The response setting selected for this study was five ("5") (approximation of absorbance) since it was found that with the stain and filter used (595 lambda) the dye appeared to obey Beer's Law. This was ascertained by testing a known sample for it's relative percentage of various fractions. For this purpose Lab-Trol was used with an albumin concentration of 67% and a known A/G ratio of 2.1. After electrophoresis and scanning the percent of albumin and A/G ratios were, as previously seen, near normal (See Table III).

7. Analysis of Absorbancy Curve of Electropherogram

a. Measurements (See Fig. 6)

The migration distance of each fraction was recorded as the distance between the point of application, and the peak of the fraction. The area under each fraction, and the total

[15]Bulletin 800-S of the Photovolt Corporation.

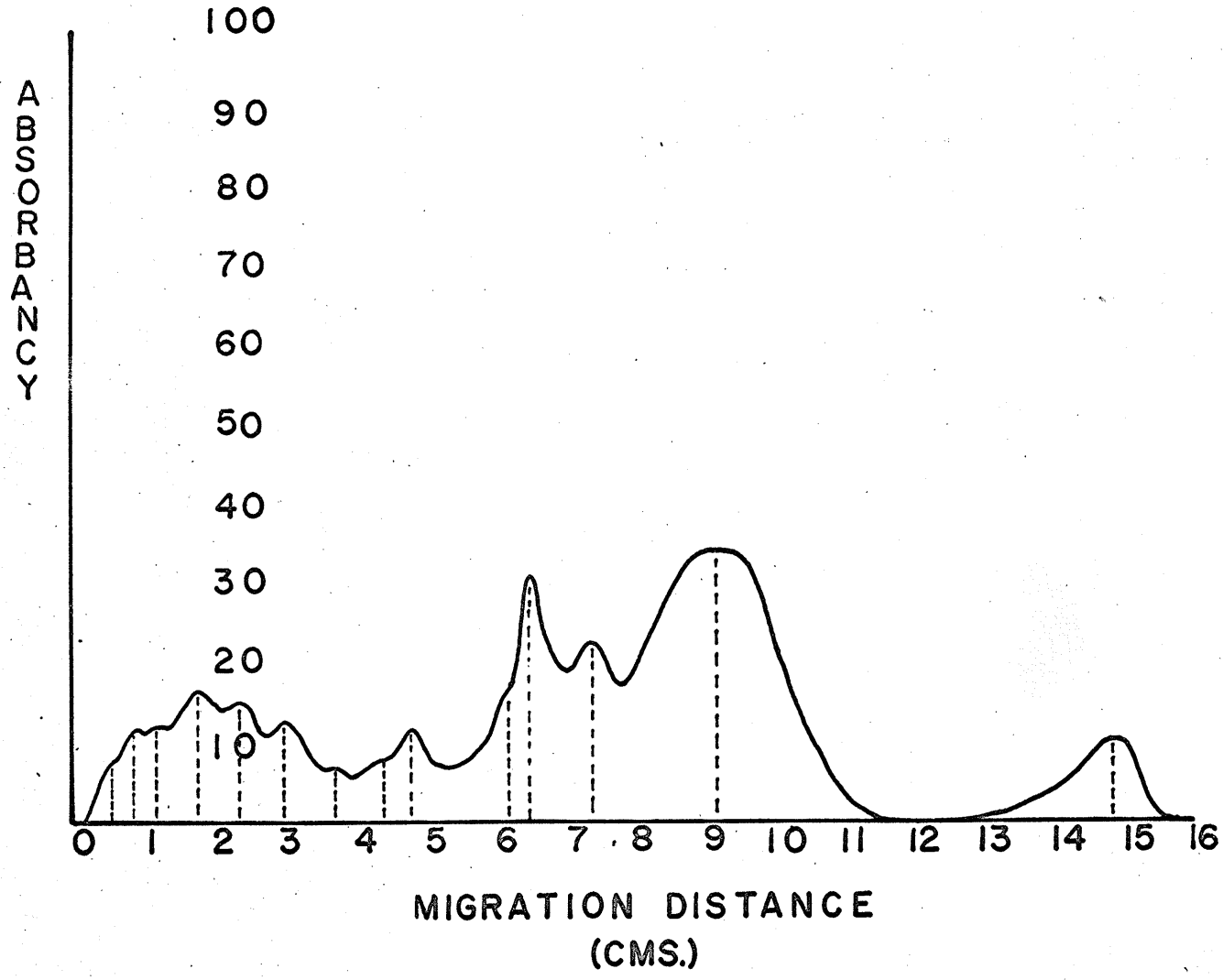

Figure 6. Absorbancy curve of electropherogram of Carassius auratus plasma protein.

area under the curve, measured planimetrically, were recorded in cm^2. In measuring these areas, the common practice of delimiting the fractions by dropping a line vertically downward from the lowest point between two neighboring fractions was used. As Labouche (1962) has pointed out, this practice introduces certain errors into the calculations. Since the area between two adjacent fractions is composed of protein from both fractions, and since each fraction has its own characteristic curve, this area may not be divided evenly between the two fractions. However, since the purpose of this experiment was not to measure exact quantities of the various plasma proteins, this feature was ignored.

As pointed out by various authorities (Smithies, 1955; Saito, 1957b; and Drilhon, 1960), it is difficult to name the various fractions without carrying out specific chemical determinations. This is caused by the facts that:

(1) Proteins with the same mobility may have entirely different fractions in different animals (Barber and Scheeler, 1963), and

(2) that the fractions obtained by paper electrophoresis are further fractionated by the starch-gel technique (Smithies, 1959b; Cooper, 1960). For this reason the system of naming, as used by Saito (1958), has been adopted. The peak with the least anodal mobility has been called fraction one, and

the others are numbered in order of increasing mobility.

b. Calculations

The total area under the curve was determined for fifty fish at each temperature with the mean, standard deviation, standard error, and 95% fiducal limits. Values were obtained for the amount under each fraction, the relative percent contributed by each fraction to the total, and the position of each fraction. Regression lines for each of these were then calculated by the least squares method, relating x to temperature, and significance tests were carried out on the difference of means, of the relative percent of each fraction, between the various experimental temperatures. The amount of each fraction was calculated by multiplying the total protein, as determined by the Biuret reaction (See below), by the relative percent of each fraction at the corresponding temperature.

8. Photographic Technique

The gels were photographed with a 35 mm camera using Ilford, Extra Fine Grain, Pan F film. The gels were placed on an x-ray viewer having a Wratten Red A safety filter and illuminated with a 25 Watt, red light. Exposure time was one second at F2. The negative was developed in Kodak developer formula D8 for three minutes at 20°C, while the positive was made with Codabromide Paper F3. After photographing the gels

were wrapped in Saran Wrap and stored in a refrigerator at 10° centrigrade.

9. Identification of the Plasma Proteins

The great majority of the fractions were characterized by their mobility relative to the mobility of the fractions in human plasma and will be discussed below. This was carried out by separating the plasma proteins of goldfish along with those from human plasma, purified human albumin, and a known control serum. The haptoglobins and lipoproteins were detected by special procedures (Smithies, 1959b) and are discussed in detail hereunder.

a. Lipoproteins

Following electrophoresis the gels were placed in fat staining solution made by mixing a saturated methanol solution of the fat dye (oil red O) with an equal volume of 20% trichloroacetic acid. The gels were left in this solution for sixteen hours. Gels were then removed and the fine deposit of solid dye on the surface of the gels was wiped off. Lipid containing fractions appeared as pink bands.

b. Haptoglobins

Following slicing, the top half of the gel was placed in a mixture of 100 ml of water, 0.5 ml glacial acetic acid, 0.2 ml hydrogen peroxide (30% w/v) and 0.2 gm of benzidine.

Hemoglobin containing bands became visible as blue areas in approximately two minutes. Photographs of the haptoglobins were obtained with a polaroid camera.

10. Total Protein Determination

Total protein was determined by the Biuret reaction using the original alkaline copper tartrate procedure as described for use with the Coleman Ultramicro Analytical program. A sixty microliter sample was added to a 12 x 75 mm cuvette and to this was added 1.2 ml of Biuret reagent[17]. The mixture was allowed to incubate for thirty minutes at the end of which 0.3 ml of 25% sodium sulfite was added in order to stop the color reaction. The optical density of the sample was then read in a Coleman Junior Spectrophotometer (Model 6C) at 555 lambda. A blank and a standard were prepared in the same way replacing the plasma with 60 microliters of distilled water and Lab Trol respectively.

Total protein was calculated according to the equation;

$$\frac{\text{optical density of "unknown total"}}{\text{optical density of standard}} \times \text{protein in standard} = \text{gms \% total protein}$$

---

[17] Biuret Reagent Tablets: Containing copper sulfate, sodium potassium tartrate, and potassium iodide. Supplied by the Cambridge Chemical Products Inc., Dearborn, Michigan.

RESULTS

Plasma protein concentration and electrophoretic patterns were determined for four groups of animals acclimated at 5°C, 12°C, 20°C, and 30°C. Data indicating the number of animals in each group, mean weight and weight range, and range of acclimmation times are summarized in Table V below.

TABLE V. Test Animals - Genera Data Showing Acclimation Times, Sample Sizes, Mean Weight and Weight Range.

Group	No. of Animals	Weight, gms. Mean	Weight, gms. Range	Acclimation Time (Days)
5°C[1]	50	25	6 - 62	26 - 44
12°C[1]	50	24	7 - 61	44 - 60
20°C[1]	50	12	6 - 22	29 - 56
30°C[1]	50	9	3 - 35	29 - 33
5°C[2]	25	31	11 - 55	26 - 44
12°C[2]	25	32	8 - 53	44 - 60
20°C[2]	25	10	6 - 22	33 - 56
30°C[2]	25	9	5 - 36	30 - 33

[1]Animals used for electrophoresis
[2]Animals used for total protein determinations

The goldfish was found to have highly coagulable and easily haemolyzed blood. In addition, the viscosity of the blood was observed to be considerably higher at the two lower test temperatures. These features rendered the collection of

unhaemolyzed plasma difficult, and many samples were discarded due to traces of haemolysis. Determination of total protein content and electrophoretic separations were normally carried out on different samples due to the small amount of plasma available from any one animal.

1. Total Plasma Protein

Due to the weight differences of the animals used, preliminary studies were carried out to determine the effect of weight on total protein content. The results of such studies were in agreement with those of Houston (unpublished data) in that the differences attributable to weight were small, and for all practical purposes may be neglected.

Values for total protein in twenty-five samples taken at each of the four acclimation temperatures are summarized in Table VI below.

TABLE VI. Total Plasma Protein as a Function of Temperature.

Plasma Protein (gms.%)	Test Temperature			
	5°C	12°C	20°C	30°C
Mean (95% Limits)	2.88±0.21	3.13±0.25	2.22±0.25	2.08±0.23
Std. Deviation	0.50	0.57	0.58	0.52
Range	1.88-3.68	2.25-4.73	0.60-3.45	0.83-3.08

P values ('t' test) less than 0.05 regarded as significant (one-tailed)

5°C to 12°C	$P > 0.05$	N.S.
5°C to 20°C	$P < 0.01$	S.
5°C to 30°C	$P < 0.01$	S.
12°C to 30°C	$P < 0.01$	S.
12°C to 20°C	$P < 0.01$	S.
20°C to 30°C	$P > 0.05$	N.S.

Regression (total protein vs temperature) $Y = 3.25 - 0.04\ X$

The mean value of the total protein content for all fish studied was 2.58 gm %, a value falling within the range of protein content reported for teleosts (see Table I). The overall decrease in the plasma protein as acclimation temperature increased from 5°C to 30°C, amounted to 27.8%, and was highly significant ($P < 0.01$). Variations between the values of total protein at the different acclimation temperatures were significant at the 0.01 level in five of the seven comparisons made (see Table VI). The remaining two, 5°C-12°C, and 20°C-30°C comparisons were not significant at the 0.05 level.

It is interesting to note that the decrease in total protein between 12°C and 20°C (3.13 - 2.22 = 0.91) was greater than that between 5°C and 30°C (2.88 - 2.08 = 0.80). This may have been the result of a chance increase between 5°C and 12°C (3.13 - 2.88 = 0.25), (see Figure 7) but most likely an accelerated decrease in total protein between 12°C and 20°C

2. Electrophoretic Analysis of the Plasma Proteins

Considerable variation was found with regard to total

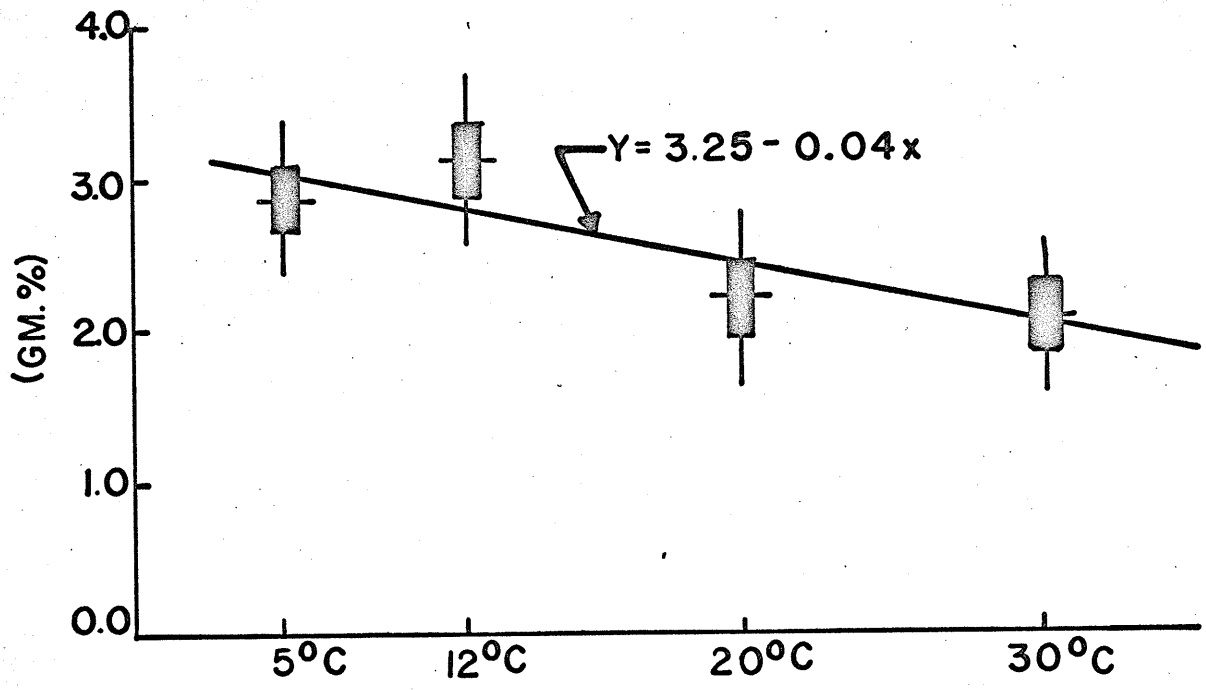

Figure 7. Variation in total protein with temperature. Vertical line represents standard deviation; horizontal line, mean; vertical bar, 95% fiducial limits of the mean.

number of fractions (see Table VII) as well as size and shape of curve (see Fig.8) in the electropaerograms of different fish.

TABLE VII. Summary of the Number of Fractions Resolved from All Fish Tested.

Group	Percent Occurance of Various Numbers of Fractions[1]							Total
	10	11	12	13	14	15	16	
5°C	6	28	28	26	8	2	1	1200
12°C	2	10	24	44	18	2	-	1272
20°C	2	14	30	24	14	12	4	1286
30°C	4	12	22	32	26	4	-	1276
All[2](Mean)	4	16	26	32	16	5	1	1259

[1]Sample size of each group was 50.
[2]Total number of fish used was 200.

Using the mean distribution of fraction number as the expected values, it was found that the distributions for the 5°C and 20°C fish differed significantly ($P < 0.01$), while those for the 12°C and 30°C fish did not ($P > 0.05$).

P values ('Chi^2' test) less than 0.05 regarded as significant.

5°C $P < 0.01$

12°C $P > 0.05$

20°C $P < 0.01$

30°C $P > 0.05$

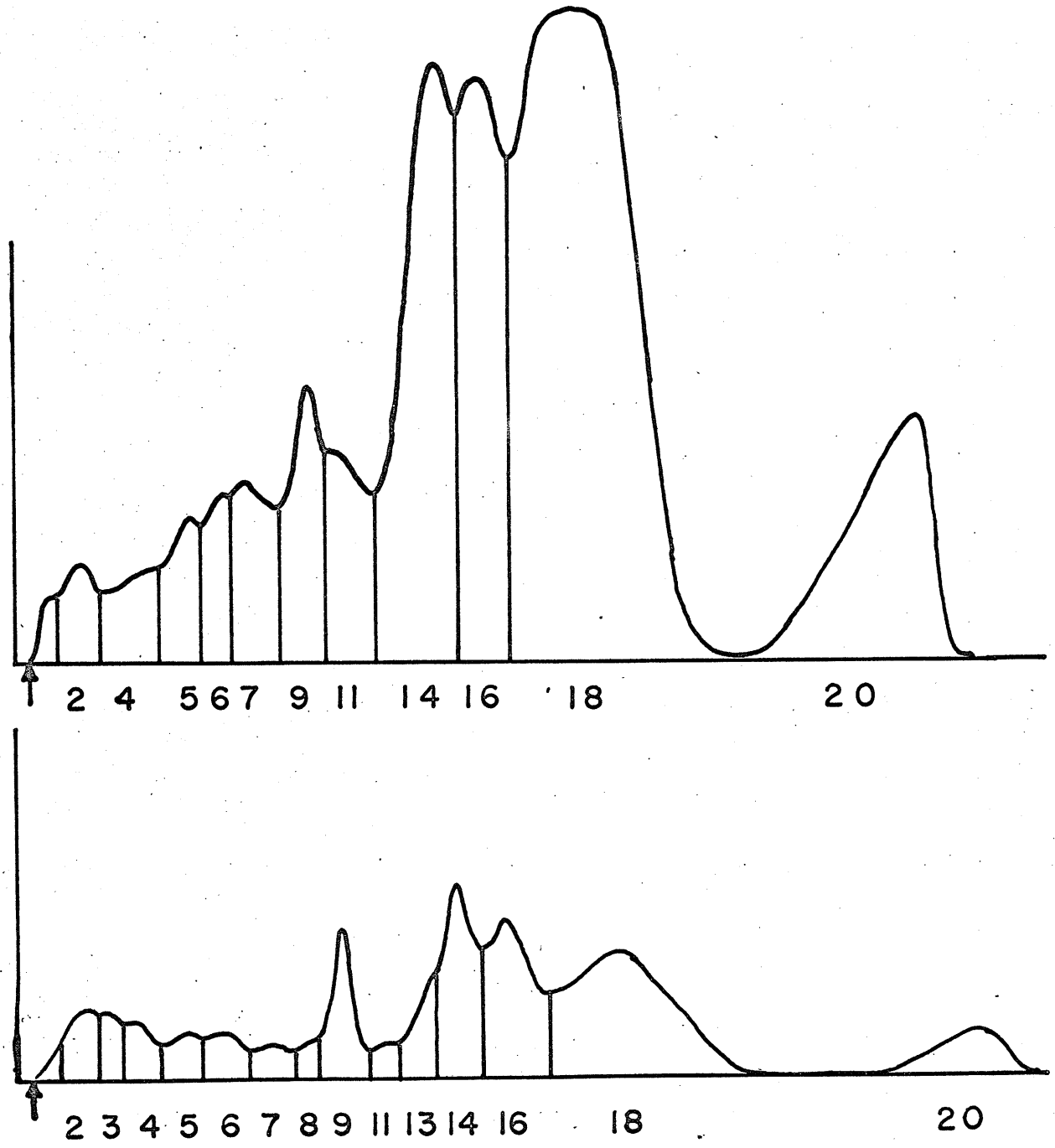

Figure 8. Possible variation in the electrophaerograms obtained at 5°C.

The number of fractions obtained by electrophoresis of any one fish ranged from 10 to 16 with an overall mean value of 13. Including all runs, a total of twenty-one seperated fractions were obtained, thirteen of which occurred in 50% to 100% of the samples (see Table VIII) and will be referred to as the typical fractions. Of these, only three appeared in 100% of the runs, suggesting the interindividual variation possible within this group of animals. As indicated in Table VII there was a slight trend toward an increase in fraction number with increased acclimation temperature (Fig.9).

The migration distances of the various fractions, measured in centimeters, may be seen in Table VIII. Contrary to Mairs and Sinderman (1960) and Thomas and McCrimmon (1964), the mobilities of the various fractions remained fairly constant from one group to another and there were no apparent significant differences. Further, in no case did any sample show even the slightest trace of a fraction with cathodal mobility although the majority had low mobilities relative to that of human albumin (Fig.10). As previously mentioned, the fractions have been numbered from one to twenty-one in order of increasing mobility.

A summary of the thermally induced changes in the total area under the electrophoretic curve, percentage of the total area under the curve contributed by each fraction, and the absolute amount of each fraction, together with the appropriate

TABLE VIII. Occurance and Mobilities of the Plasma Proteins of the Goldfish

Fraction	Mobility (cms.)				Occurance (%)			
	5°C	12°C	20°C	30°C	5°C	12°C	20°C	30°C
1	0.3	-	0.3	-	4	-	6	-
2*	0.6	0.6	0.6	0.6	94	100	98	100
3*	1.0	1.2	1.1	1.0	72	58	60	72
4*	1.6	1.7	1.7	1.6	96	90	80	84
5*	2.2	2.2	2.3	2.3	98	100	98	98
6*	2.8	2.8	2.8	2.8	96	100	98	98
7*	3.4	3.4	3.4	3.4	74	96	90	92
8*	4.0	4.1	4.1	4.0	62	76	76	62
9*	4.5	4.7	4.6	4.6	100	100	100	100
10	4.9	-	4.9	4.8	4	-	12	16
11*	5.1	5.2	5.0	64	64	44	48	50
12	5.4	5.8	5.5	5.5	14	24	20	28
13	6.1	6.2	6.3	6.2	26	36	44	42
14*	6.4	6.6	6.6	6.6	90	90	88	96
15	6.8	7.0	6.9	6.9	18	26	38	24
16*	7.0	7.1	7.4	7.2	86	92	90	92
17	7.7	8.1	7.9	7.9	22	22	30	24
18*	8.6	8.6	9.0	8.6	100	100	100	100
19	-	10.8	-	-	-	12	-	-
20*	14.2	14.6	14.3	14.0	100	100	100	100
21	-	16.5	-	-	-	6	-	-

* Fractions considered to be typical.

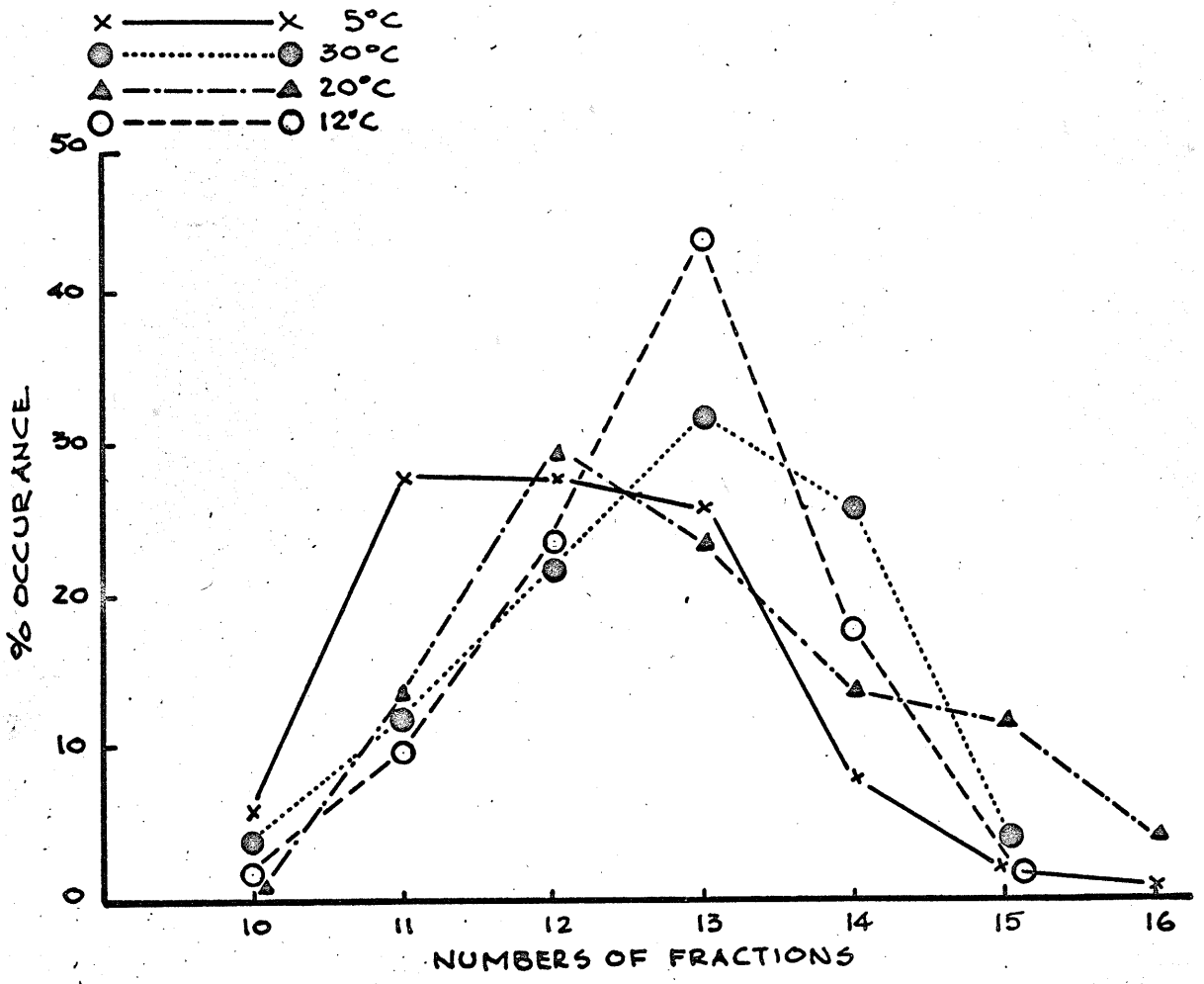

Figure 9. Variation in fraction abundance with temperature.

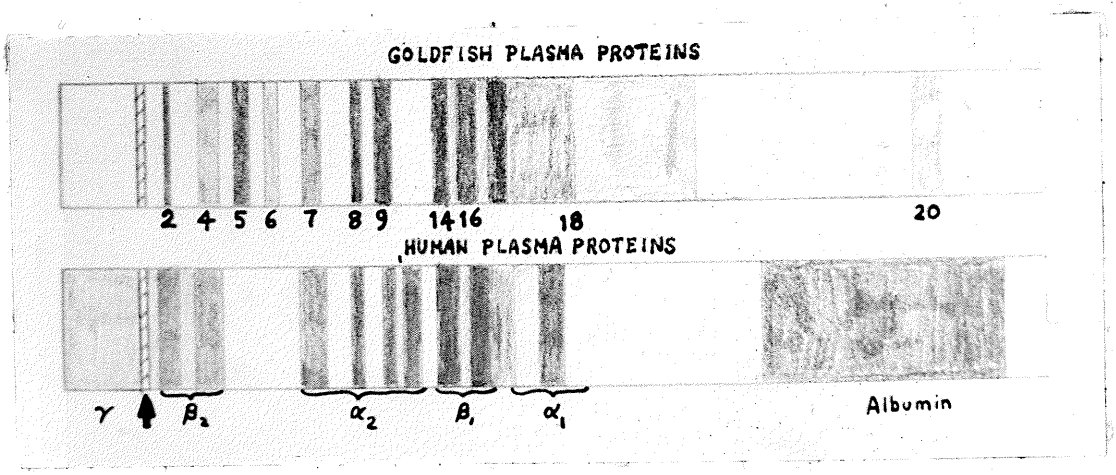

Figure 10. Photographic Comparison of the Electrophoretic Pattern of A, Goldfish Plasma, and B, Human Plasma.

information regarding significance of means and regression against temperature will be found in detail below.

a. Total Area Under the Curve

The total area under the curve was considered approximately proportional to the total stainable protein in the sample and was used as a rough index of the protein concentration.

The mean total area under the curve for all samples was 28.9 cm^2 (S = 13.7 cm^2, 95% fiducial limits 28.9 ± 1.9, St, error = 1.0 cm^2). In accordance with the total protein determinations (Table VI), total area under the curve showed a decrease with temperature from 36.1 cm^2 at 5°C to 25.7 cm^2 at 30°C (Table IX), the difference being significant at the 0.01 level.

TABLE IX. Differences and Significance of Differences Between the Total Area Under Curve at Different Acclimation Temperatures.

Total Area Under Curve (cm^2)	5°C	12°C	20°C	30°C
Mean (95% limits)	36.1±4.3	29.7±3.1	24.2±3.1	25.7±3.5
Std. Deviation	15.6	11.6	11.7	12.4
Std. Error	2.2	1.6	1.6	1.8
Range	12.7-61.3	8.6-60.4	8.6-58.0	5.6-58.0

P values ('z' test) less than 0.05 regarded as significant (one-tailed).

5°C - 12°C	$P < 0.01$	12°C - 20°C	$P < 0.1$
5°C - 20°C	$P < 0.01$	12°C - 30°C	$P < 0.01$
5°C - 30°C	$P < 0.01$	20°C - 30°C	$P > 0.10$

All inter-group differences of means, with the exception of 20° - 30°C, were found to be significant at the 0.01 level. The regression of total area under curve against temperature was $Y = 36.0 - 0.42 X$ (see Fig.11).

b. The Individual Fractions

Of the twenty-one fractions recognized in goldfish plasma, eight were present in less than 50% of the runs, and were not considered to be typical constituents (Table VIII). Each fraction may be characterized by a mean mobility (Table VIII) and a mean size (Fig.12). This figure also illustrates the method of numbering fractions.

Data for typical fractions from all runs in each temperature group is presented in Table X, and includes the means, 95% fiducial limits, and standard deviations of the percentage of the total area under the curve contributed by each fraction, as well as the absolute amount (mean relative percent x mean total protein) of each fraction.

Table XI shows, in addition to the regressions of relative percent contributed by each fraction to temperature and absolute amount of each fraction to temperature, the significance

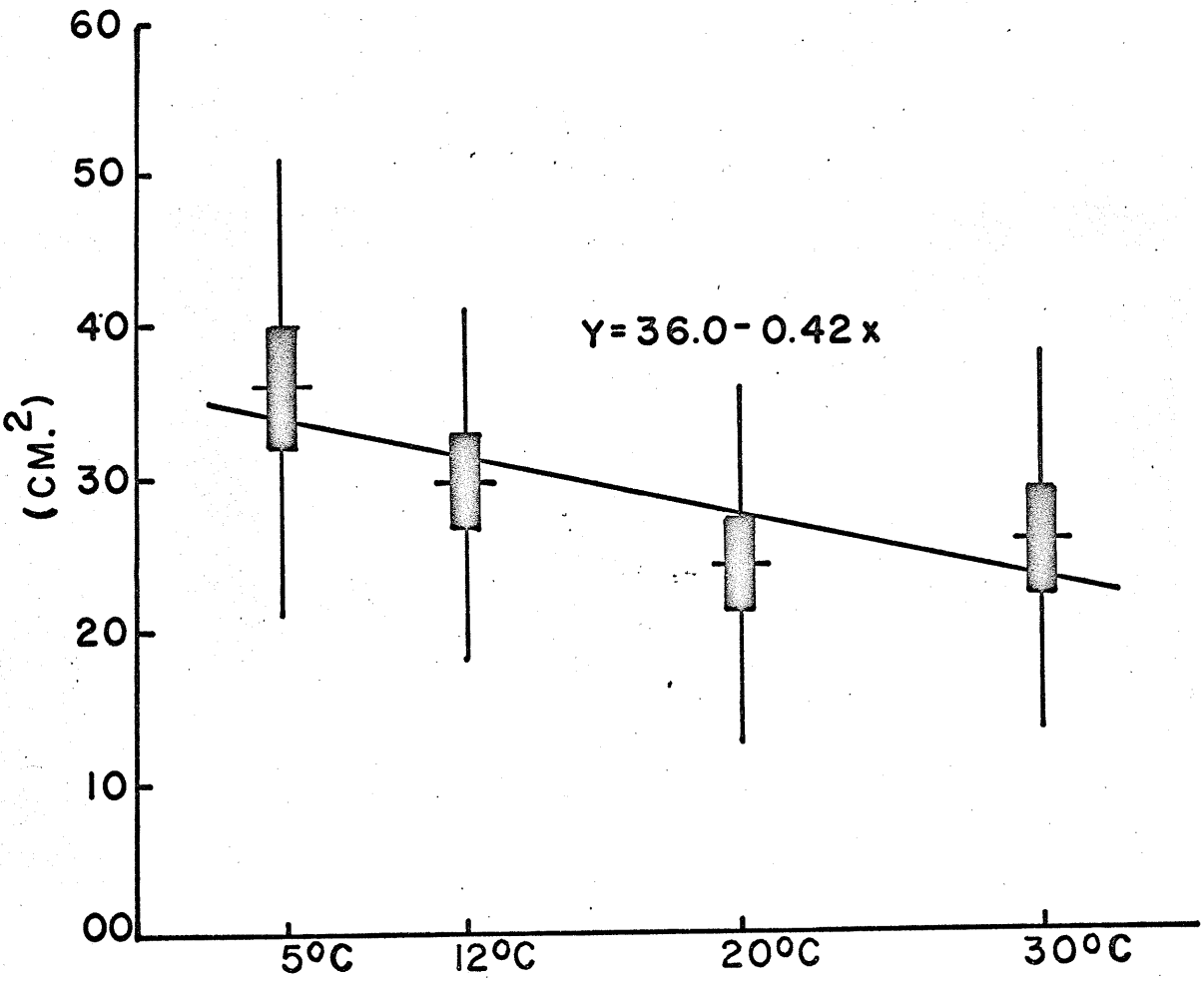

Figure 11. Variation in the total area under the electrophoretic curve with temperature. Vertical line represents standard deviation; horizontal line, mean; vertical bar, 95% fiducal limits of the mean.

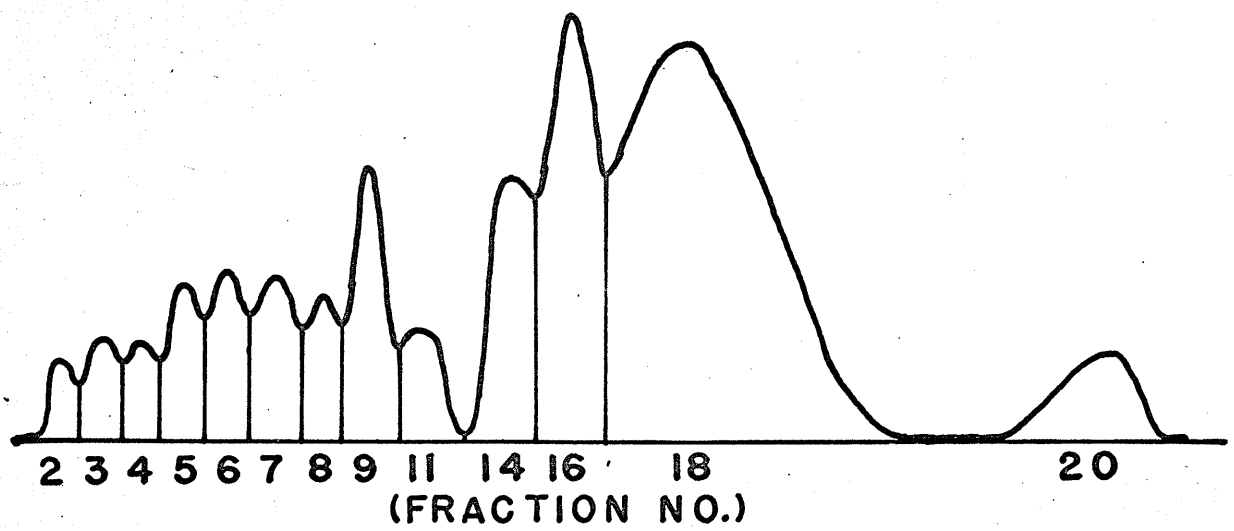

Figure 12. Curve showing all typical peaks at average size.

TABLE X. Mean Absolute Amount and Percent Contribution to the Total Area Under the Curve of Each Fraction.

Fraction	Group	Mean with 95% Fiducal Limits (% Contribution)	Standard Deviation (% Contribution)	Absolute Amount (mg.%)
2	5°C	1.8 ± 0.39	1.4	50
	12°C	1.3 ± 0.25	0.9	40
	20°C	2.3 ± 0.51	1.8	50
	30°C	2.9 ± 0.61	2.2	60
3	5°C	2.6 ± 0.74	2.3	70
	12°C	2.0 ± 0.43	1.1	60
	20°C	3.3 ± 0.78	2.2	70
	30°C	3.4 ± 0.74	2.3	70
4	5°C	3.1 ± 0.47	1.7	90
	12°C	2.3 ± 0.41	1.4	70
	20°C	3.3 ± 0.68	2.2	70
	30°C	3.1 ± 0.54	1.8	60
5	5°C	3.6 ± 0.45	1.6	100
	12°C	2.9 ± 0.39	1.4	90
	20°C	3.5 ± 0.47	1.7	80
	30°C	3.2 ± 0.47	1.7	70

continued

TABLE X (Continued)

Fraction	Group	Mean with 95% Fiducal Limits (% Contribution)	Standard Deviation (% Contribution	Absolute Amount (mg.%)
6	5°C	4.1 ± 0.82	2.9	120
	12°C	3.2 ± 0.55	2.0	100
	20°C	4.0 ± 0.47	1.7	90
	30°C	3.3 ± 0.41	1.5	70
7	5°C	4.1 ± 0.94	2.9	120
	12°C	3.2 ± 0.51	1.8	100
	20°C	3.5 ± 0.53	1.8	80
	30°C	3.5 ± 0.41	1.4	70
8	5°C	2.8 ± 0.27	0.8	80
	12°C	3.2 ± 0.55	1.7	100
	20°C	3.5 ± 0.73	2.3	80
	30°C	3.2 ± 0.53	1.5	70
9	5°C	7.0 ± 0.53	1.9	200
	12°C	8.4 ± 0.80	2.9	260
	20°C	6.4 ± 0.53	1.9	140
	30°C	4.9 ± 0.53	1.9	100
11	5°C	3.8 ± 0.51	1.5	110
	12°C	3.6 ± 0.81	1.8	110
	20°C	3.3 ± 0.79	1.8	70
	30°C	3.1 ± 0.60	1.4	60

continued

TABLE X (Continued)

Fraction	Group	Mean with 95% Fiducal Limits (% Contribution)	Standard Deviation (% Contribution	Absolute Amount (mg.%)
14	5°C	9.9 ± 1.18	4.0	280
	12°C	8.1 ± 1.29	4.4	250
	20°C	7.9 ± 1.18	4.0	180
	30°C	8.5 ± 0.96	3.4	180
16	5°C	12.6 ± 1.49	5.0	360
	12°C	12.3 ± 2.55	8.8	380
	20°C	9.3 ± 1.25	4.3	210
	30°C	10.2 ± 1.10	3.8	210
18	5°C	39.9 ± 2.35	8.5	1150
	12°C	39.5 ± 3.33	12.0	1240
	20°C	41.4 ± 3.23	11.7	920
	30°C	39.3 ± 2.86	10.3	820
20	5°C	6.3 ± 1.10	3.8	180
	12°C	5.5 ± 0.92	3.3	170
	20°C	7.4 ± 1.22	4.4	160
	30°C	9.8 ± 1.27	4.6	200

TABLE XI. Relationship Between Relative and Absolute Amounts of Each Fraction to Temperature.

Fraction	Regression (a) Relative % to temperature (b) Absolute amt. to temp.gm.%	Significance of inter-group difference of means ('z' or 't' test)[1]					
		5-12	5-20	5-30	12-20	12-30	20-30
2	(a) Y=1.18 + 0.05x	S	NS	VS	VS	VS	NS
	(b) Y=0.04 + 0.0005x						
3	(a) Y = 2.06 + 0.04x	NS	NS	NS	VS	VS	NS
	(b) Y=0.065 + 0.0001x						
4	(a) Y=2.74 + 0.013x	VS	NS	NS	VS	S	NS
	(b) Y=0.09 - 0.001x						
5	(a) Y=3.41 - 0.007x	S	NS	NS	S	NS	NS
	(b) Y=0.10 - 0.001x						
6	(a) Y=3.97 - 0.02x	S	NS	S	NS	NS	NS
	(b) Y=0.13 - 0.002x						
7	(a) Y=3.85 - 0.016x	NS	NS	NS	NS	NS	NS
	(b) Y=0.13 - 0.002x						
8	(a) Y=2.90 + 0.016x	NS	S	NS	NS	NS	NS
	(b) Y=0.093 - 0.0006x						
9	(a) Y=8.4 - 0.10x	VS	NS	VS	VS	VS	S
	(b) Y=0.26 - 0.005x						

continued

[1] P values greater than 0.05 are regarded as not significant (NS), less than 0.05 but greater than 0.01 as significant (S), and less than 0.01 as very significant (VS).

TABLE XI (Continued)

Fraction	(a) Regression Relative % to temperature (b) Absolute amt. to temp.gm.%	Significance of inter-group difference of means ('z' or 't' test)					
		5-12	5-20	5-30	12-20	12-30	20-30
11	(a) Y=3.93 - 0.029x	NS*	NS*	S*	NS*	NS*	NS*
	(b) Y=0.126 - 0.002x						
14	(a) Y=9.4 - 0.05x	S	VS	S	NS	NS	NS
	(b) Y=0.30 - 0.004x						
16	(a) Y=13.08 - 0.12x	NS	VS	VS	S	NS	NS
	(b) Y=0.41 - 0.007x						
18	(a) Y=40.08 - 0.003x	NS	NS	NS	NS	NS	NS
	(b) Y=1.30 - 0.02x						
20	(a) Y=4.65 + 0.16x	NS	NS	VS	VS	VS	VS
	(b) Y=0.16 + 0.0007x						

* 't' test results

of all inter-group differences of means. This data is also illustrated in Figures 13-26 inclusive, along with the mean percent of total globulin, regression of total percent of the curve contributed by globulin against temperature, mean values of the albumin to globulin ratio, and the regression of albumin to globulin ratio with temperature.

It was found that of the thirteen typical fractions, the relative percent showed a significant decrease ($P < 0.05$) in six,

FIGURE 13. Variation in total plasma protein (A), relative (B), and absolute amounts (C) of fraction 2 with temperature.

Ordinate A: Total protein, gm.%
Ordinate B: Relative percent of total protein contributed by Fraction 2.
Ordinate C: Absolute amount, gm.%

Vertical lines represent standard deviations; horizontal lines, mean; vertical bars, 95% fiducial limits; equations, regression against temperature.

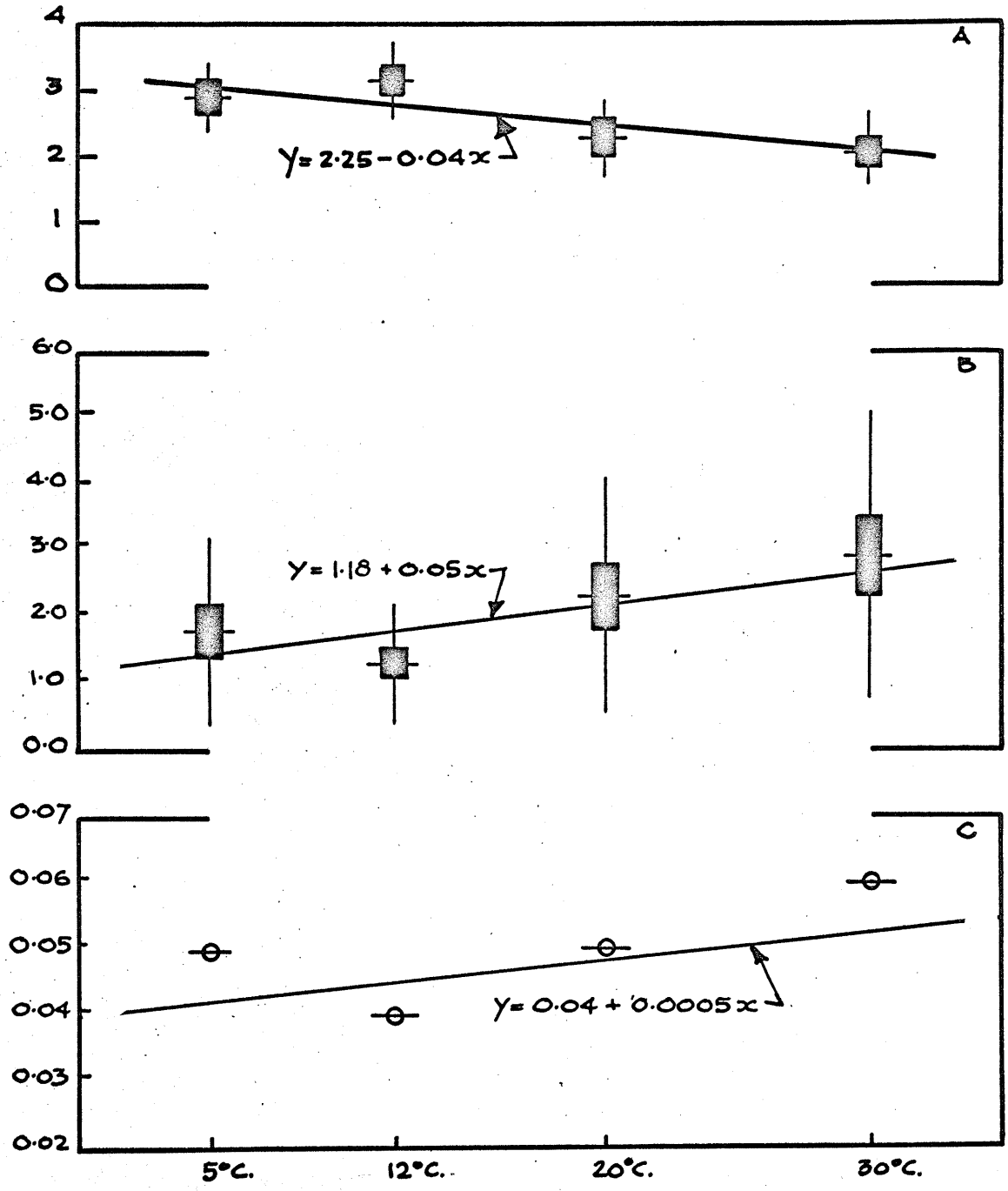

Figure 13

FIGURE 14. Variation in total plasma protein (A), relative (B), and absolute amounts (C) of fraction 3 with temperature.

Ordinate A: Total protein, gm.%
Ordinate B: Relative percent of total protein contributed by fraction 3.
Ordinate C: Absolute amount, gm.%

Vertical lines represent standard deviation; horizontal lines, mean; vertical bars, 95% fiducal limits; equations, regression against temperature.

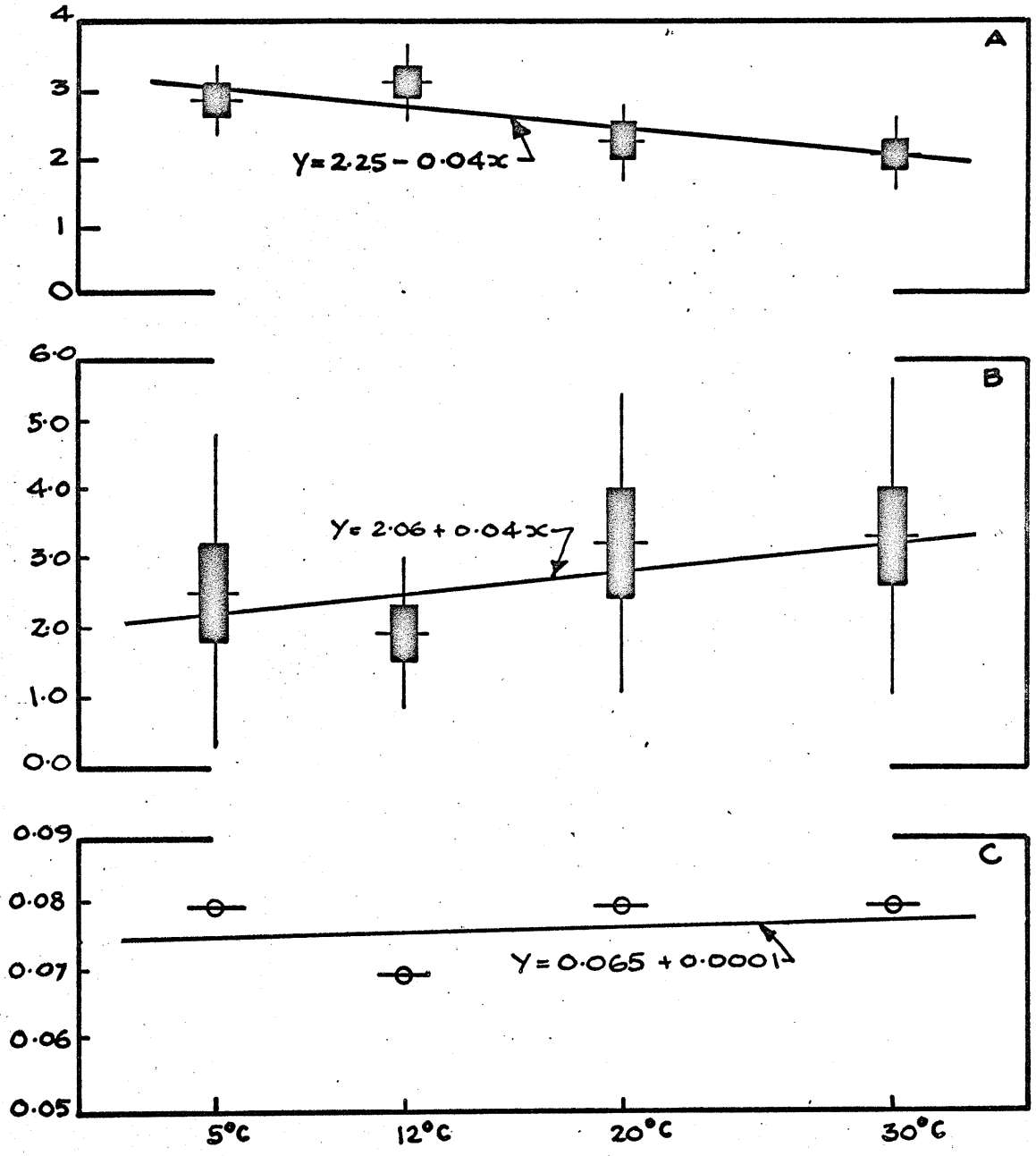

Figure 14

FIGURE 15. Variation in total plasma protein (A), relative (B), and absolute amounts (C) of fraction 4 with temperature.

Ordinate A: Total protein, gm.%
Ordinate B: Relative percent of total protein contributed by fraction 4.
Ordinate C: Absolute amounts, gm.%

Vertical lines represent standard deviation; horizontal lines, mean; vertical bars, 95% fiducial limits; equations, regression against temperature.

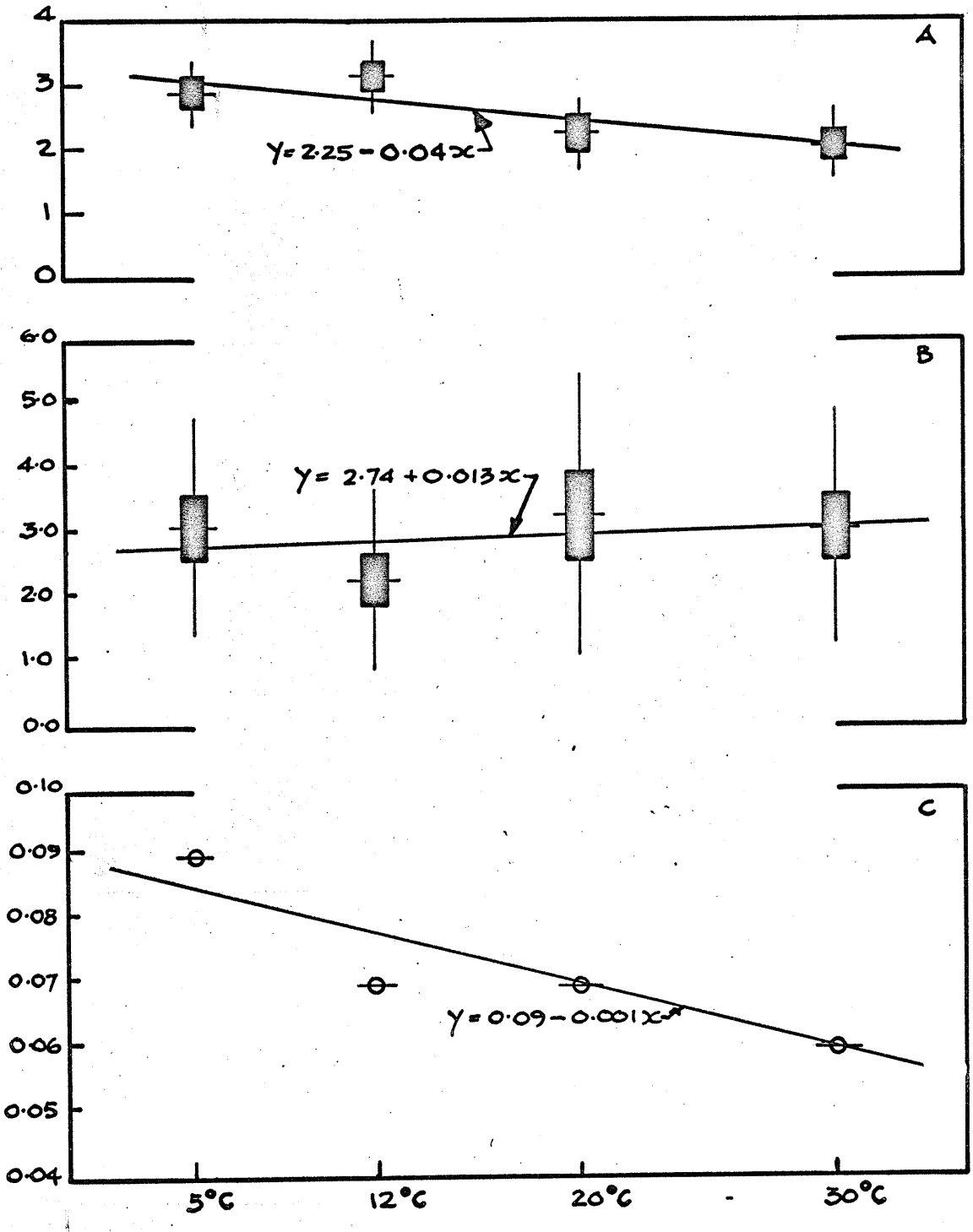

Figure 15

FIGURE 16. Variation in total plasma protein (A), relative (B), and absolute amounts (C) of fraction 5 with temperature.

Ordinate A: Total protein, gm.%
Ordinate B: Relative percent of total protein contributed by fraction 5.
Ordinate C: Absolute amounts, gm.%

Vertical lines represent standard deviation; horizontal lines, mean; vertical bars, 95% fiducial limits; equations, regression against temperature.

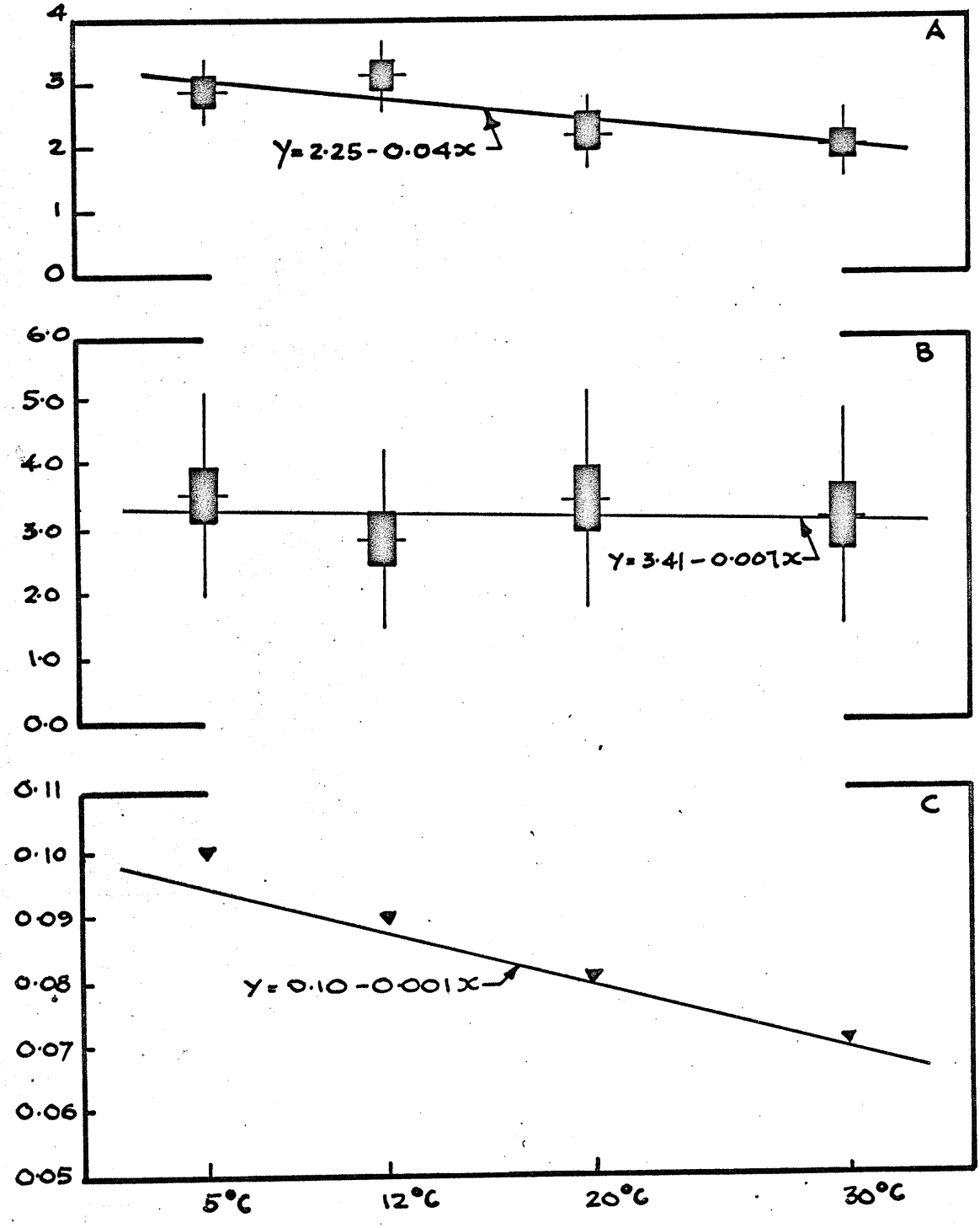
A
4
3
2
1
0
Y= 2·25 - 0·04x
B
6·0
5·0
4·0
3·0
2·0
1·0
0·0
Y= 3·41 - 0·007x
C
0·11
0·10
0·09
0·08
0·07
0·06
0·05
Y = 0·10 - 0·001x
5°C
12°C
20°C
30°C

Figure 16

FIGURE 17. Variation in total plasma protein (A), relative (B), and absolute amounts (C) of fraction 6 with temperature.

Ordinate A: Total protein, gm.%
Ordinate B: Relative percent of total protein contributed by fraction 6.
Ordinate C: Absolute amounts, gm.%

Vertical lines represent standard deviation; horizontal lines, mean; vertical bars, 95% fiducial limits; equations, regression against temperature.

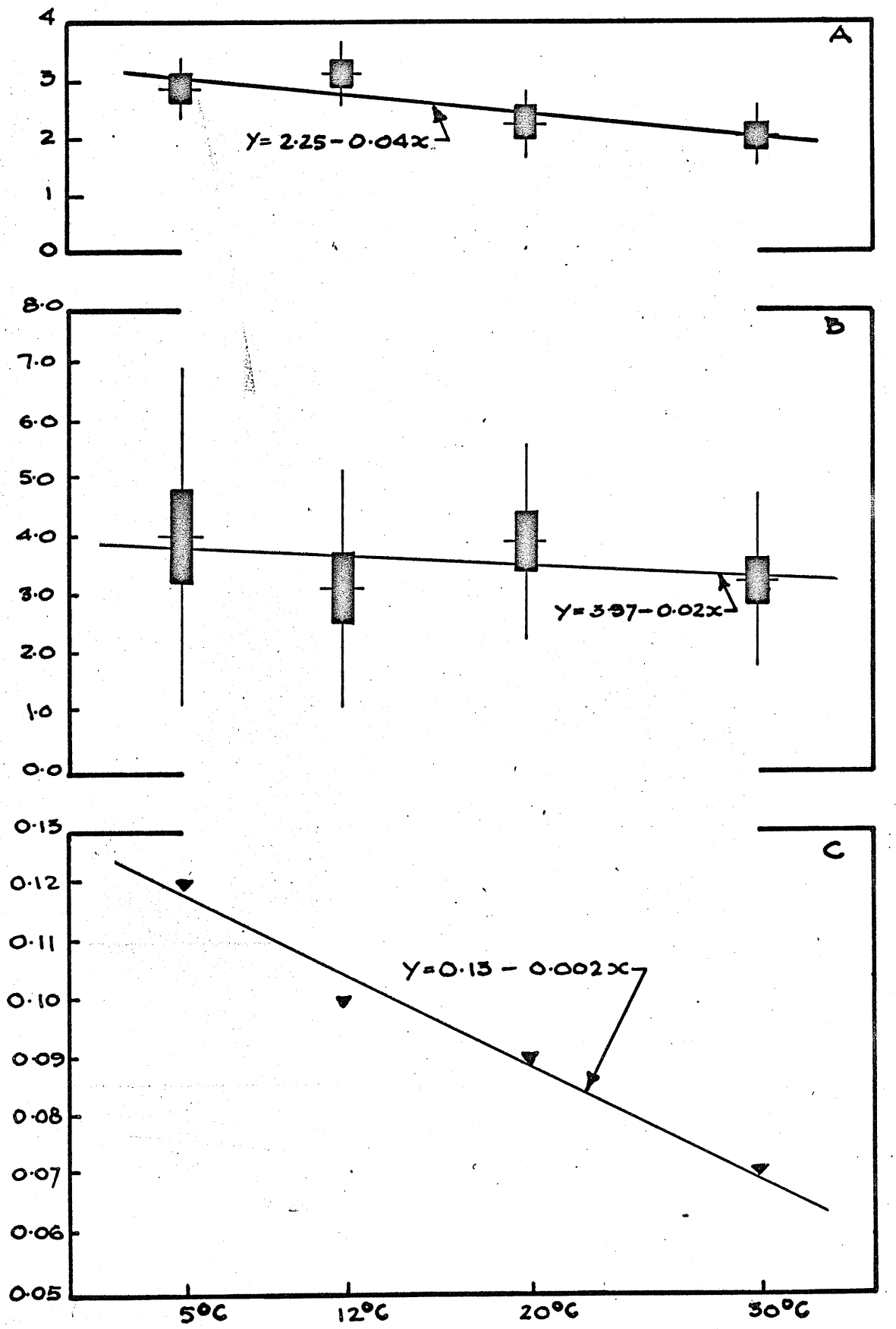

Figure 17

FIGURE 18. Variation in total plasma protein (A), relative (B), and absolute amounts (C) of fraction 7 with temperature.

Ordinate A: Total protein, gm.%
Ordinate B: Relative percent of total protein contributed by fraction 7.
Ordinate C: Absolute amount, gm.%

Vertical lines represent standard deviation; horizontal lines, mean; vertical bars, 95% fiducial limits; equations regression against temperature.

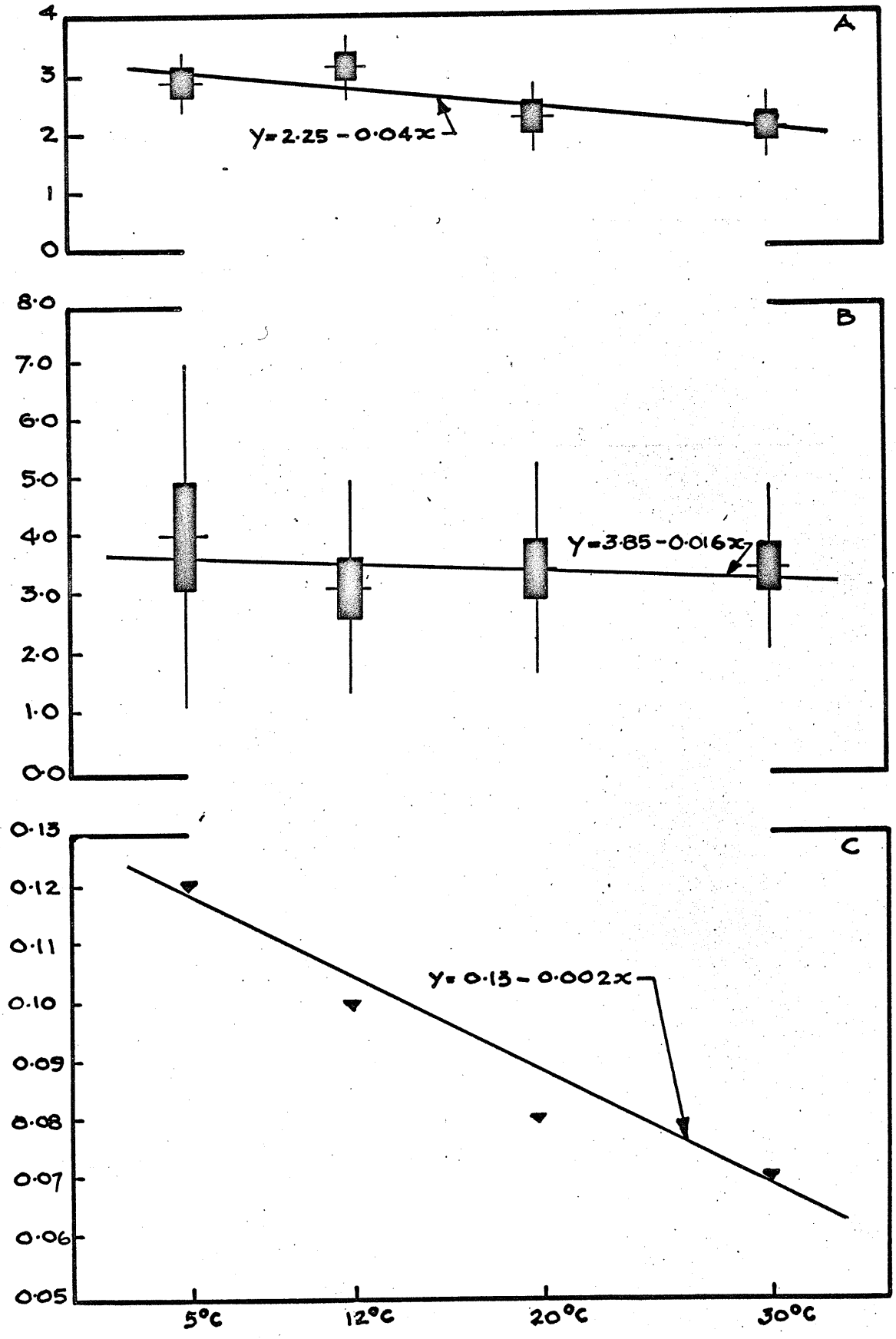

Figure 18

FIGURE 19. Variation in total plasma protein (A), relative (B), and absolute amounts (C) of fraction 8 with temperature.

Ordinate A: Total protein, gm. %
Ordinate B: Relative percent of total protein contributed by fraction 8.
Ordinate C: Absolute amount, gm. %

Vertical lines represent standard deviation; horizontal lines, mean; vertical bars, 95% fiducial limits; equations, regression against temperature.

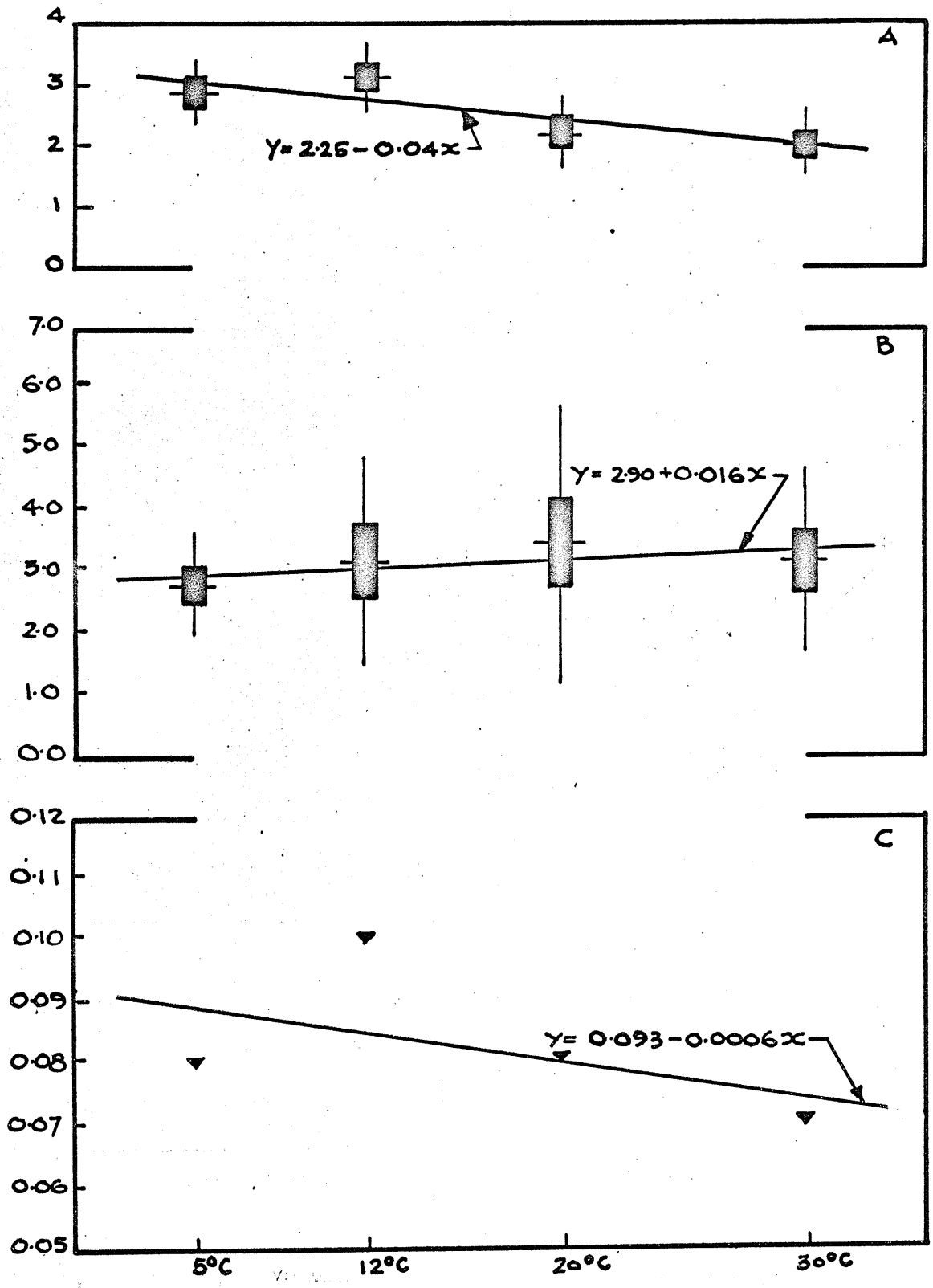

Figure 19

FIGURE 20. Variation in total plasma protein (A), relative (B), and absolute amounts (C) of fraction 9 with temperature.

Ordinate A: Total protein, gm. %
Ordinate B: Relative percent of total protein contributed by fraction 9.
Ordinate C: Absolute amount, gm.%

Vertical lines represent standard deviation; horizontal lines, mean; vertical bars, 95% fiducial limits; equations, regression against temperature.

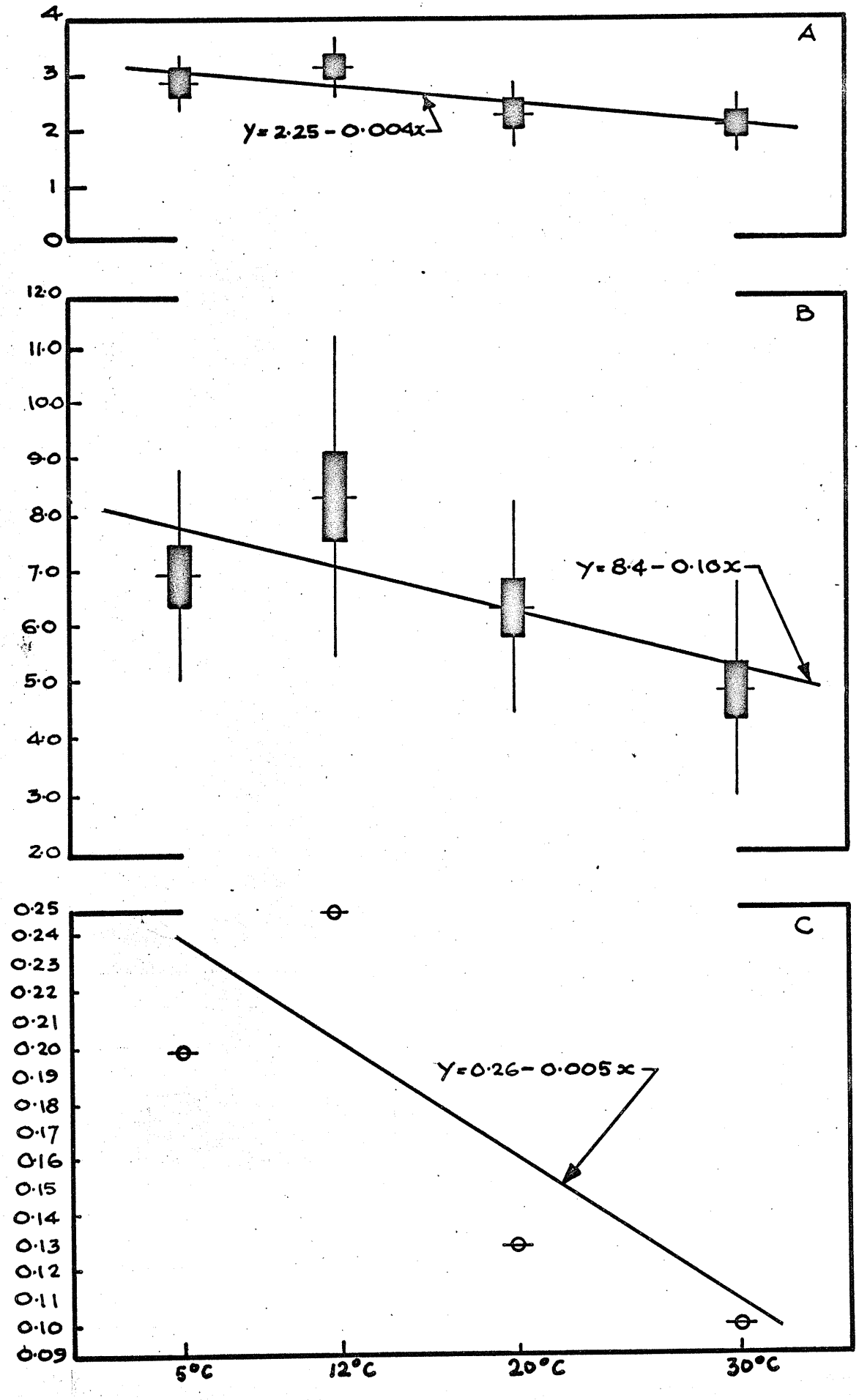

Figure 20

FIGURE 21. Variation in total plasma protein (A), relative (B), and absolute amounts (C), of fraction 11 with temperature.

Ordinate A: Total protein, gm.%
Ordinate B: Relative percent of total protein contributed by fraction 11.
Ordinate C: Absolute amount, gm.%

Vertical lines represent standard deviation; horizontal lines, mean; vertical bars, 95% fiducial limits; equations, regression against temperature.

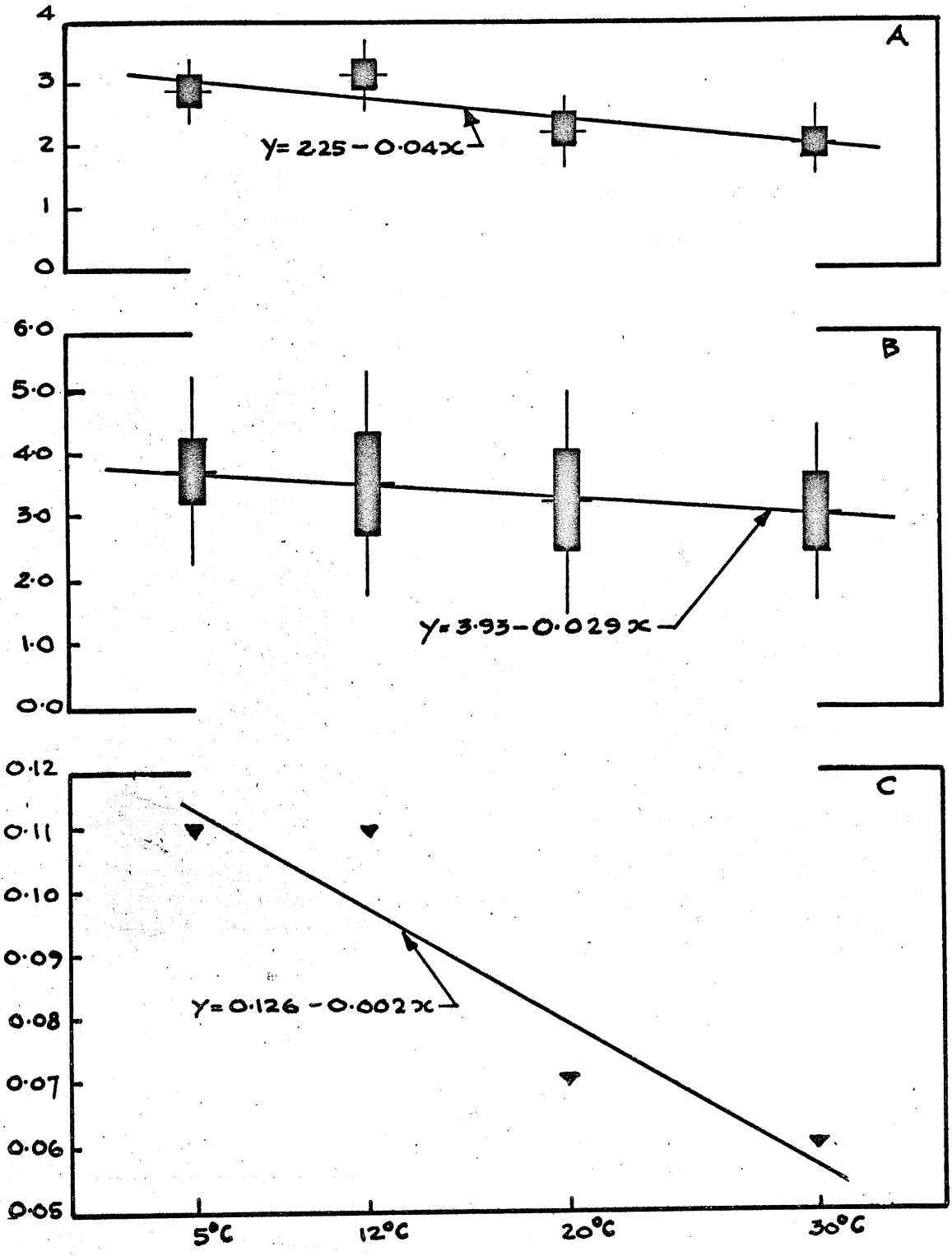

Figure 21

FIGURE 22. Variation in total plasma protein (A), relative (B), and absolute amounts (C) of fraction 14 with temperature.

Ordinate A: Total protein, gm.%
Ordinate B: Relative percent of total protein contributed by fraction 14.
Ordinate C: Absolute amount, gm.%

Vertical lines represent standard deviation; horizontal lines, mean; vertical bars, 95% fiducial limits; equations, regression against temperature.

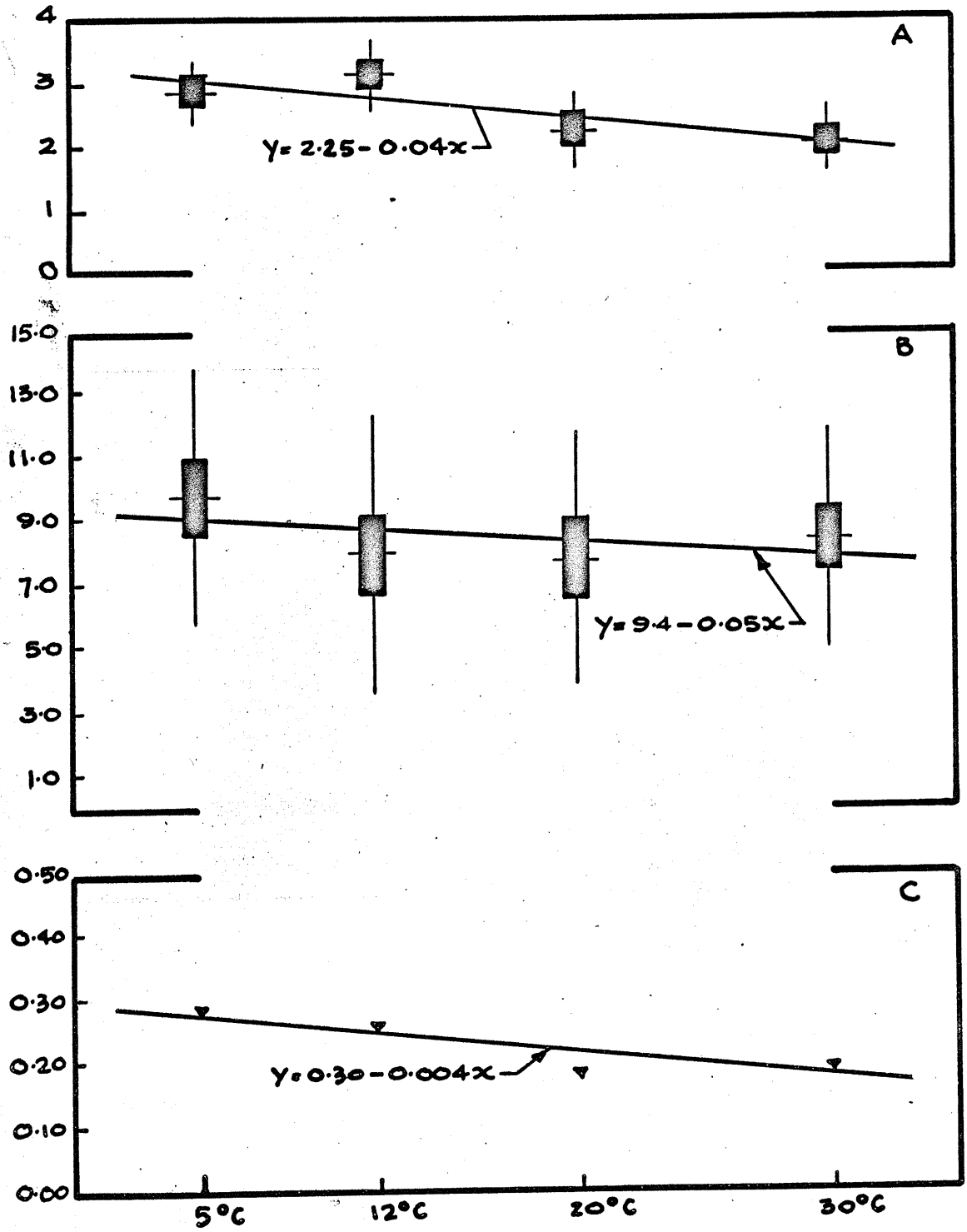

Figure 22

FIGURE 23. Variation in total plasma protein (A), relative (B), and absolute amounts (C) of fraction 16 with temperature.

Ordinate A: Total protein, gm. %
Ordinate B: Relative percent of total protein contributed by fraction 16.
Ordinate C: Absolute amount, gm.%

Vertical lines represent standard deviation; horizontal lines, mean; vertical bars, 95% fiducial limits; equations, regression against temperature.

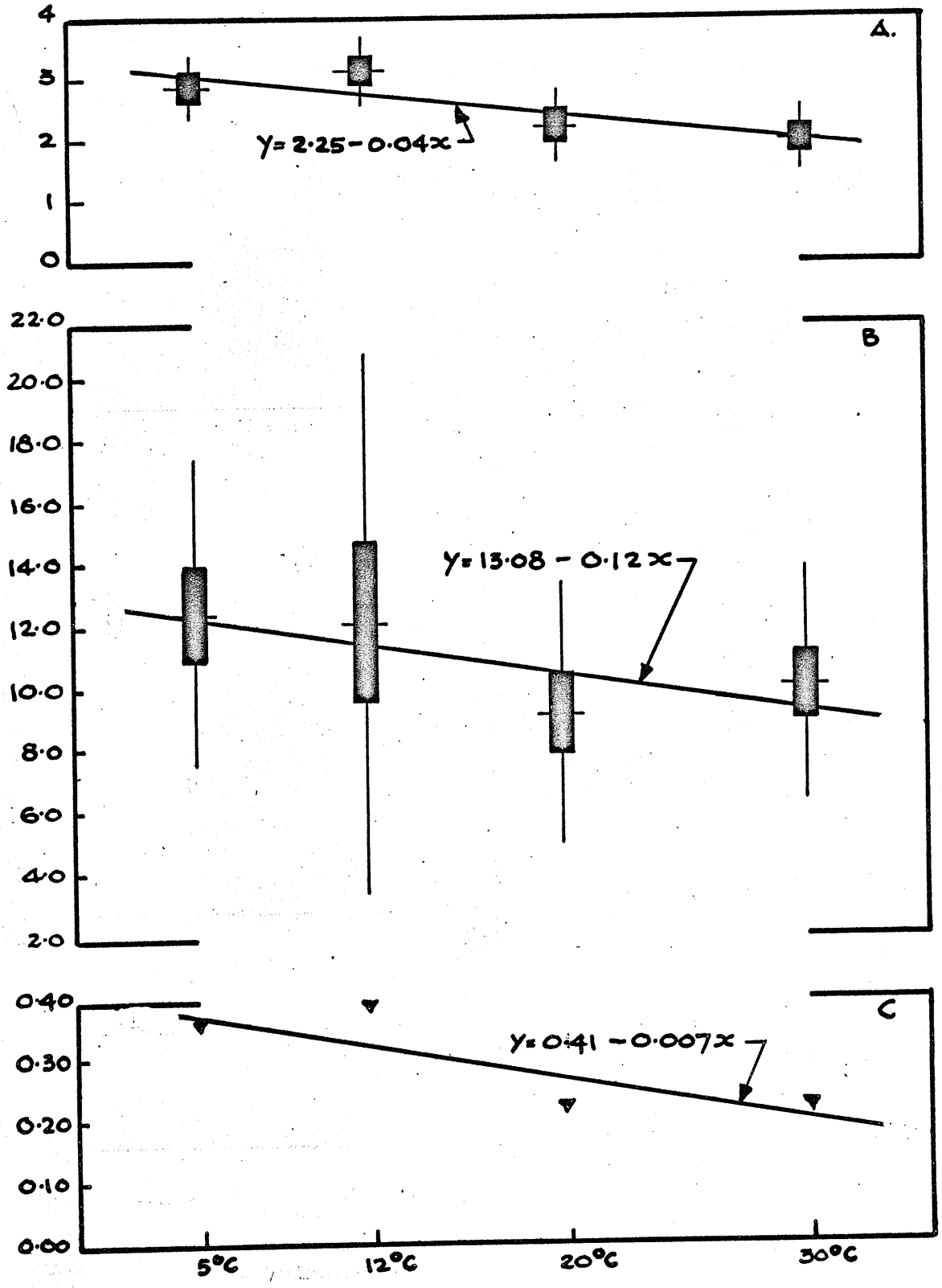

Figure 23

FIGURE 24. Variation in total plasma protein (A), relative (B), and absolute amounts (C) of fraction 18 with temperature.

Ordinate A: Total protein, gm. %
Ordinate B: Relative percent of total protein contributed by fraction 18.
Ordinate C: Absolute amount, gm.%

Vertical lines represent standard deviation; horizontal lines, mean; vertical bars, 95% fiducial limits; equations, regression against temperature.