ABSTRACT

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BOVINE, PORCINE, AND AVIAN GLYCOPROTEINS - CHEMICAL COMPOSITIONS, ELECTROPHORETIC DISTRIBUTIONS, AND INCORPORATION OF INJECTED C\textsuperscript{14}-GLUCOSAMINE

Statistically significant species differences were found in the levels of hexose, hexosamine and fucose. Porcine sera contained more hexose and fucose than did the others, while avian sera contained more hexosamine.

Species differences were evident in the distributions of protein and carbohydrates following electrophoresis on paper, cellulose acetate, polyacrylamide and agar.

The electrophoretic distributions of protein were not identical for the media employed.

The P-2, P-3 and P-4 fractions, obtained by a sequential precipitation of plasma proteins with ammonium sulphate followed by chromatography on CM-cellulose, appear to contain different glycoproteins.

Glucosamine-1-C\textsuperscript{14} reached maximum levels in avian plasma proteins 2 to 10 hours following intravenous injection. The activity appears to be preferentially bound by some proteins.
Suggested short title:

BOVINE, PORCINE AND AVIAN GLYCOPROTEINS

M. CHANNON
BOVINE, PORCINE, AND AVIAN GLYCOPROTEINS -
CHEMICAL COMPOSITIONS, ELECTROPHORETIC
DISTRIBUTIONS, AND INCORPORATION
OF INJECTED C\textsuperscript{14}-GLUCOSAMINE

by

Michael Channon

A thesis submitted to the Faculty of Graduate Studies and
Research in partial fulfilment of the requirements for the
degree of Master of Science

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GENERAL INTRODUCTION

The presence of carbohydrate containing proteins in the blood has been known for many years. Considerable interest has been devoted to the glycoproteins in recent years, especially in humans, due to their great physiological significance.

In recent years this Department has been concerned with the glycoproteins of some domestic animals, including the bovine, porcine and avian species. A study has been made of the electrophoretic distribution of protein and hexosamine following paper electrophoresis and also of sialic acid following agar electrophoresis.

The M-1 and M-2 glycoproteins from bovine, porcine and avian plasma were isolated in purified forms and were partially characterized.

Preliminary experiments were conducted to study the incorporation of $^{14}$C-glucosamine into avian plasma proteins.

The present thesis is concerned with:
(a) An investigation of variability of the hexosamine determination.
(b) An extension of the previous studies on electrophoretic distribution to include distribution in cellulose
acetate and polyacrylamide.

(c) The mapping of the distribution of hexose, hexosamine, sialic acid and fucose among the fractional ammonium sulphate precipitates and among the fractions isolated by carboxymethyl cellulose chromatography.

(d) A study of the incorporation of glucosamine-1-C\textsuperscript{14} into the avian plasma proteins.
I. LITERATURE REVIEW

A. INTRODUCTION

The presence of carbohydrate in combination with plasma proteins was demonstrated as early as the 1890's by Freund (1), Morner (2) and Zanetti (3). In 1903 Zanetti (4) established the presence of glucosamine in serum.

B. NOMENCLATURE

The term glycoprotein was originally used to describe protein molecules containing carbohydrate substance or substances other than nucleic acids (5).

Levene in 1925 (6) proposed a system of nomenclature dividing the carbohydrate containing protein compounds or mucoproteins into chondroitin sulphuric acids and the mucoitin sulphuric acids. This classification was based on the false assumption that all mucoproteins were sulphated and contained uronic acid.

Meyer (7,8,9) proposed and later modified a system of classification. He divided the hexosamine containing substances into mucopolysaccharides, mucoids and glycoproteins, based on their uronic acid and hexosamine contents. According to Meyer (7) glycoproteins were substances which contained
a mucopolysaccharide in firm chemical union with protein, containing less than four per cent carbohydrate. In 1953 (9) he subdivided the glycoproteins into mucoproteins and mucoids, the former having an easily split carbohydrate to protein linkage while the latter had a firmer linkage.

Jeanloz (10) proposed omission of the term "muco" from the nomenclature. The carbohydrate-protein complexes were to be differentiated in terms of the linkage into either polysaccharide-protein complexes, bound by a weak linkage, or glycoproteins, bound by a strong covalent linkage.

Winzler (11) employed a similar system but limited the term glycoprotein to those complexes containing greater than 1% hexose bound covalently.

Gottschalk (12) has proposed a more precise definition for glycoprotein. He defined as glycoprotein a conjugated protein having as a prosthetic group one or more heterosaccharides characterized by a relatively small number of sugar residues which lack a serially repeating unit and are bound covalently to the protein chain.

C. CARBOHYDRATE UNIT

Dische in 1928 (13) demonstrated the presence of D-mannose in proteins of horse plasma. He also suggested the
possibility of there being an unidentified glucosamine present as well.

The presence of mannose in horse, dog, donkey and bird plasma, and of D-glucose, D-glucosamine, D-mannose and D-galactose in protein bound carbohydrate was demonstrated by Bierry (14-17).

Rimington (18) observed the presence of a trisaccharide containing one mole of glucosamine and two moles of mannose in horse serum proteins.

Goa in 1955 (19) demonstrated the presence of D-galactosamine in horse serum proteins.

Odin in 1955 (20) demonstrated the presence of sialic acid in human serum proteins.

The presence of fucose in serum proteins was demonstrated by Dische and Shettles (21).

The sugars shown to occur in the carbohydrate units of glycoprotein are then the hexoses, galactose and mannose; the hexosamines, glucosamine and galactosamine; as well as fucose and sialic acid.

The most common sialic acids are N-acetyl neuraminic acid and N-glycolylneuraminic acid (22). Other sialic acids have been observed including O-acetyl, N, O-diacetyl and O-methyl neuraminic acids (23).
It is important to note, however, that many glycoproteins do not contain all of these residues. Fibrin (24) and fibrinogen (25) appear to be free of fucose. Others (26, 27) are free of sialic acid and still others are free of galactosamine (28).

The sialic acids are the sugars which contribute most to the chemical and physical properties of the glycoproteins in which they are contained. This is due to the carboxyl group of the sialic acid (pK$_a$ 2.6) (29) in addition to the five hydroxyl and one acetamido group.

Of equal importance is the fact that the sialic acid usually is in the terminal position on the carbohydrate chain. For example in the case of fetuin, Oshiro and Eyler (30) have shown that removal of sialic acid raises the isoelectric point from pH 4.23 to pH 5.57. They did not find removal of sialic acid resulted in conformational changes on the fetuin molecule.

Although the foregoing sugars are the major ones found in glycoproteins, others have been found. Weicker (31) reported the isolation of a glycoprotein from the stroma of erythrocytes containing 3.5% xylose.

These sugars are combined to form oligosaccharides units which are the carbohydrate moieties of the glycoproteins. The carbohydrate units may vary in size from
monosaccharides up to large heterosaccharides. Spiro (32) reported that characterization of the carbohydrate components of fetuin, thyroglobulin and glomerular basement membranes revealed components varying in size from monosaccharides to heteropolysaccharides of up to 17 sugars.

Glycoproteins may occur with either one kind of carbohydrate unit per molecule or with a number of different ones per molecule. Rosevear and Smith (33) working on a glycoprotein from the γ-globulin fraction of human plasma, were able to isolate the carbohydrate moiety (M.W. 3600) consisting of five or six mannoses, three galactoses, six glucosamines, two fucoses and one sialic acid, from the glycoprotein which had a molecular weight of 157,000 and carbohydrate content of 1.2 per cent. They deduced that there could be only one such heterosaccharide per molecule.

Many other glycoproteins have been shown to contain more than one heterosaccharide residue per molecule. Recently Bennich and Johansson (34) have reported on the structure of the immunoglobulins, noting the immunoglobulin ND possibly contains six carbohydrate units per molecule, three per heavy chain. Gerbeck, Bezkovainy and Rafelson (35) studied haptoglobin types 2-1 and 2-2 from human plasma. It was reported that the former had up to 13 units per
molecule and the latter up to 26 units per molecule.

Gottschalk (36) working on the urinary glycoprotein of Tamm and Horsfall (37,38) showed it to contain from 75 to 300 heterosaccharide residues per molecule; each residue consisting of eight hexosamines, four galactose, three mannose, three sialic acids and one fucose.

In almost all cases the terminal unit of the carbohydrate moiety is sialic acid. This has been demonstrated by treating glycoprotein with receptor destroying enzyme (RDE), a neuramidase isolated from *Vibrio Cholerae* shown to cleave the O-glycosidic linkage between sialic acid and N-acetyl galactosamine (39-41).

Gottschalk and Lind (42) treating ovomucin with RDE noted a loss in biological activity, increase in pH and decrease in electrophoretic mobility. From the digest, a low molecular weight compound, identified as sialic acid was isolated.

Pope and Maxfield (43) treated the human urinary glycoprotein of Tamm and Horsfall with neuramidase and found that three of the four neuraminic acids were released.

It is thought that sialic acid is linked to galactose and N-acetyl galactose by an O-glycosidic bond (40,41).

Spiro and Spiro (44) isolated and characterized a
sialyltransferase which catalyzed the transfer of CMP-N-acetylneuraminic acid to glycopeptide and glycoprotein acceptors. The best acceptor molecules were desialyzed glycoprotein molecules, with free galactose residues. The enzyme did not transfer sialic acid to the N-acetyl derivative of galactose.

Though sialic acid occupies the terminal position of the carbohydrates unit and does affect the physical and chemical properties of the molecule, it does not appear to have any effect on molecular conformation. Yamagami and Schmid (45) showed that the presence of sialyl residues did not appreciably influence the conformation of the polypeptide chain. Oshiro and Eyler (30) have shown that there are no conformational changes associated with the removal of sialyl residues.

Thus in most glycoproteins the terminal sugar is sialic acid and the next sugar is galactose.

The linkage of the carbohydrate moiety to the polypeptide chain has been an area of intensive investigation. There appear to be three major types of linkages (46). The most frequent linkages are N-glycosidic linkages between C-1 of N-acetylglucosamine and the amide of asparagine. This type of linkage appears to be common to most serum proteins with
the exception of those in the albumin region. A second type of linkage is an O-glycosidic linkage involving the C-1 of the sugar and the hydroxyl group of serine and threonine. The sugar is usually N-acetylgalactosamine.

The third type of linkage is a glycosidic ester linkage involving N-acetylgalactosamine and the ω-carboxyl of aspartic or glutamic acid.

Recently Moczar (47), Bahl (48) and Wagh, Bornstein and Winzler (49) have all reported N-glycosidic linkages between N-acetylglucosamine and aspartic acid. Bahl (48) also reported an N-glycosidic linkage between N-acetylgalactosamine and serine in human chorionic gonadotrophin.

Bhargava, Buddecke, Werries and Gottschalk (50) have demonstrated an O-glycosidic bond between N-acetylgalactosamine and serine or threonine in an ovine submaxillary gland glycoprotein.

Morgan (51) demonstrated the presence of an O-glycosidic linkage of carbohydrate to serine or threonine in blood group specific glycoproteins.

Dawson and Clamp (52) have reported that a human myeloma globulin possibly contains two different carbohydrate peptide linkages in the same molecule. These are a N-glycosidic linkage involving galactosamine and aspartic acid and
an O-glycosidic linkage involving serine or threonine.

Spik, Monsigny and Montreuil (53) have demonstrated the presence of an N-glycosidic linkage of aspartic acid and N-acetyl galactosamine and an O-glycosidic linkage of threonine with galactose or mannose, in human lactotransferrin.

Enzymes responsible for the transfer of sugar residues to amino acids in polypeptide chains have been isolated. McGuire and Roseman (54), Hagopian, Bosmann and Eylar (55), Hagopian and Eylar (56,57) have all reported the isolation of transferases which catalyze the transfer of N-acetylgalactosamine from its UDP derivative to hydroxy-amino acids.

The nature of the protein components have not been studied as extensively as has the nature of the carbohydrate moieties. While the quantities of individual amino acids may readily be determined by automated amino acid analyzers and by gas-liquid chromatography, determination of the sequence of amino acids is much more difficult. Complicating this procedure is the fact that the sequence need not be the same at different sites of carbohydrate bonding, even within the same molecule.

For example, Haschemeyer, Cynkin, Han and Tridle (58) have determined the amino acid sequence of glycopeptides
obtained from bovine fibrinogen. Bovine fibrinogen was digested with pronase to release glycopeptides which were isolated and purified by gel filtration and ion-exchange chromatography. Three glycopeptides, containing up to 80 per cent of the carbohydrate were isolated and their amino and sequence determined. These were:

(1) $H_2N\text{-Asp-Hys-COOH}$

(2) $H_2N\text{-Gly-Glu-Asp-Arg-COOH}$

(3) $H_2N\text{-Glu-Asp-Arg-COOH}$

Gerbeck et al. (35) digested haptoglobin 2-1 and 2-2 with pronase and isolated the glycopeptides. One glycopeptide which contained aspartic acid, alanine, serine and threonine with aspartic acid and glutamic acid N-terminal was isolated. A second glycopeptide containing aspartic acid and histidine with both N-terminal was also isolated.

The secondary and tertiary structures of the glycoprotein have not been investigated to a great extent. Pope and Maxfield (43) working on the human urinary glycoprotein of Tamm and Horsfall (37,38) found that as sialic acid was released by a neuramidase there was a corresponding increase in the number of titratable arginine groups and an increase
in the number of tyrosine groups with a pK lower than in the native state. It was suggested that each sialic acid molecule may be linked to tyrosine by hydrogen bonding.

Krotoski and Weimer (59) treated human plasma orosomucoid with mercaptoethanol and iodoacetamide and 8 M urea. From their results they concluded that there may be a possible -S-S- dependent tertiary structure to the glycoprotein. They proposed several possible polypeptide backbone structures ranging from a simple U-shaped single polypeptide chain to a S-shaped chain of five polypeptides linked by carbohydrate.

D. PLASMA GLYCOPROTEIN

Most research in plasma glycoproteins has dealt with those of the human. Comparatively little research has been carried out concerning the plasma glycoproteins of animals. Some general surveys have been made as to the blood concentration of characteristic glycoprotein constituents and to their electrophoretic distribution in conjunction with the electrophoretic distribution of plasma proteins (22, 60-74). Furthermore, acid glycoproteins (75-77) and fetuin (24, 78-81) of domestic animals have been investigated.
1. Acid Glycoproteins

Acid glycoproteins are constituents of all mammalian species studied (75). The acid glycoproteins may be separated into two components, M-1 and M-2, by electrophoresis at pH 4.5 (82).

α1-acid Glycoprotein

α1-acid glycoprotein, also known as orosomucoid, seromucoid, α1-acid seromucoid, orosomucoid and M-1 glycoprotein, is one of the most significant and well characterized glycoproteins, both of human and animal plasma. The isolation of this glycoprotein from human plasma was originally described by Weimer, Wehl and Winzler (83) and by Schmid (84). Since then it has been isolated from bovine plasma by Bezkorovainy and Doherty (76) and from bovine, porcine and avian plasma by Grant, Martin and Anastassiadis (77). Grant et al. reported similar properties for their M-1 and M-2 glycoproteins as were reported by Bezkorovainy and Doherty (76). It was noted that the sedimentation coefficients \( S_{20,w}^0 \) for their glycoproteins were somewhat lower than those reported by Bezkorovainy and Doherty. Grant et al. found different molecular weights and extinction coefficients than Bezkorovainy and Doherty.

Grant et al. noted that the M-1 glycoproteins did not
exhibit as much polymorphism as did human α1-acid glycoprotein. Pronounced species differences were reported by Grant et al. in the amino acid composition of the M-1 and M-2 glycoproteins. Arginine, threonine, serine, proline, valine, isoleucine, leucine and phenylalanine contents of porcine M-1 glycoprotein were lower and lysine and cystine contents were higher than in bovine M-1 glycoprotein. Also lysine, aspartic acid, isoleucine and tyrosine contents of avian M-1 glycoprotein were found to be lower and histidine contents were found to be higher than in the bovine and porcine M-1 glycoproteins. As well, the aspartic acid, threonine, proline, alanine, valine, leucine and phenylalanine contents of porcine M-2 glycoprotein were higher and glutamic acid, cystine and tyrosine contents were lower than in bovine M-2 glycoprotein.

The amino acid composition differed from that reported by Bezkorovainy and Doherty (76,85), primarily in aspartic acid and leucine.

Grant et al. did not report any pronounced species variation in hexosamine content of the glycoproteins of the three species investigated. The most striking difference reported was in fucose contents. Bovine and avian M-1 and M-2 glycoproteins contained little fucose, whereas, porcine
M-1 and M-2 glycoproteins contained 25.3 per cent and 14.8 per cent, respectively. They concluded that porcine M-1 and M-2 glycoproteins must be more branched than bovine M-1 and M-2 glycoproteins.

Bezkorovainy and Doherty (85) also studied some apparent physiological properties of the bovine M-2 glycoproteins. They noted that the M-2 glycoprotein bound thyroxine more readily than did human serum albumin. The M-2 glycoprotein did not have any apparent hemoglobin binding properties.

Bezkorovainy (86) in studying the acid glycoproteins of bovine plasma reported that sialic acid was present as both the N-glycolyl and N-acetyl neuraminic acids, with slightly more of the N-glycolyl form. Glucosamine appeared to be the only hexosamine present. Galactose and mannose were the only two hexoses. Both proteins appeared to contain equimolar amounts of galactose and mannose, though in the case of M-2 glycoproteins there was some evidence of more galactose. These results agree with those reported by Grant et al. (77).

2. \( \alpha_2 \)-Globulins

The \( \alpha_2 \)-globulin fraction of the serum contains at least three major glycoproteins: ceruloplasmin, haptoglobin
and prothrombin. As stated before little research has been conducted on these glycoproteins in animal blood plasma.

3. Ceruloplasmin

Osaki (87,88) reported a method of obtaining crystalline porcine ceruloplasmin of molecular weight 162,000 containing both Cu$_2^{+2}$ and Cu$^{+2}$. Koya, Osaka and Sato (89) demonstrated that porcine ceruloplasmin was a phenylalanine-phenylalanine peptide. They proposed a glycopeptide which contained the sugars mannose, galactose, glucose and xylose and the amino acids threonine, alanine and lysine and histidine. It was postulated that the carbohydrate moiety was attached to the ε amino of lysine.

Jamieson (90) studying human ceruloplasmin reported a more conventional structure. The carbohydrate component of human ceruloplasmin appears to consist of heterosaccharide chains of molecular weight 1150-1250 with possibly 8-10 such chains per molecule. All sialic acid was found to be terminal occurring in five or six of these chains. The constituent chains are not likely of identical composition. The carbohydrate chains are likely joined to the protein at aspartic acid.
4. **Haptoglobin**

The haptoglobin are genetically determined serum $\alpha_2$-glycoproteins which exhibit the unique property of binding hemoglobin to form a stable complex (35). The major genetic forms are identified as Hp 1-1, 2-1 and 2-2.

Human haptoglobin has been shown to have a carbohydrate component containing 3.1% N-acetyleneuraminic acid, 5.8% glucosamine, 0.6% fucose and 7.5% hexose (91). Cheftel (92) has reported a composition of 5.1% N-acetyleneuraminic acid, 5.4% glucosamine, 1% fucose, 0.5% glucose and 8.5% galactose and mannose.

There appears to be two polypeptide chains in the haptoglobin molecule (93), an $\alpha$-chain which varies with genetic type of haptoglobin and a $\beta$-chain which is common to all types (94,95). It appears that the carbohydrate is associated with the $\beta$-chain. Shim and Bearn (96) have shown that all sialic acid is associated with the $\beta$-chain which contained 7.3% to 7.6% sialic acid. Shim and Bearn also reported that in type 1-1 haptoglobin there appeared to be two $\alpha$- and two $\beta$-chains per molecule, joined by $-S-S-$ bonds. Recently Giblett (97) has reviewed research on human haptoglobins.
5. **Prothrombin**

Prothrombin is an $\alpha_2$-glycoprotein involved in the process of blood clotting. The carbohydrate has been identified as containing 4.5% hexose (98) and 1.6% hexosamine (99). Sialic acid is also present (100). The probable molecular weight is in the area of 62,700 for bovine prothrombin (99). Prothrombin is physiologically active in the clotting of blood, during which process 60 to 80% of the carbohydrate and 40% of the nitrogen is split off (101).

6. **$\beta$-globulin**

Two physiologically important glycoproteins are found in the $\beta$-globulin region. These are transferrin and fibrinogen. Other $\beta$-globulin glycoproteins have also been isolated (27).

i. **Transferrin**

Transferrin (102), also known as siderrophilin or $\beta$, metal containing protein (103) is an iron binding $\beta$-globulin. It is characterized by a carbohydrate content of 5 - 6% in humans. The carbohydrate moiety consists of four moles sialic acid, eight moles mannose, four moles galactose and eight moles of glucosamine per molecule (104). The molecular weight of transferrin has been established as 90,000.
(105-107) and does not appear to contain fucose. Jamieson (104) also reports that the carbohydrate appears to be linked to the protein by an asparaginylglycosylamine linkage. Spik et al. (53) have confirmed this but have also demonstrated the presence of O-threonylglycosidic bonds. The carbohydrate moiety is probably branched in structure with sialic acid the terminal, non-reducing residue.

The protein has been identified as being a single chain of 750 amino acid residues (109).

Transferrin is physiologically active as a means of iron transport in the blood (109,110). Two molecules of trivalent iron may be reversibly bound to a transferrin molecule. Aasa, Malmstrom, Saltman and Vangard (111) have suggested that the binding is by chelation. It has been suggested that the iron is binding with three tyrosine and two histidine residues (112). Windle et al. (112) and Aasa et al. (111) have also suggested the involvement of two imidazole residues. Cu$^{+2}$ and Zn$^{+2}$ may also be bound by transferrin but with less affinity than Fe$^{+3}$ (111).

ii. Fibrinogen

Fibrinogen is the other major -glycoprotein. Bovine fibrinogen has been isolated and characterized by Davie and Ratnoff (113). They have reported that it is a
molecule composed of six polypeptide subunits, containing 3 per cent carbohydrate, and of molecular weight 340,000. The carbohydrate contains 17 N-acetylglucosamine, 7 galactose, 14 mannose and 7 N-acetylneuraminic acid. Haschemeyer et al. (58) reported that these residues are grouped in six or seven oligosaccharides, likely linked to aspartic acid and asparagine.

Fibrinogen is physiologically active in the blood clotting mechanism. Fibrinogen is converted to fibrin, losing up to 20 per cent of its carbohydrates in the process (114-116).

Schmid et al. (27) have reported the isolation of a \( \beta_1 \)-glycoprotein, characterized by its lack of sialic acid, from human plasma. The protein, isolated from Cohn Fraction VI, had a molecular weight of 30,000, isoelectric point at pH 4.4 and isoionic point at pH 4.9. The carbohydrate, comprising 30 per cent of the total molecular weight contained galactose, mannose, fucose and glucosamine.

7. \( \gamma \)-globulins

The \( \gamma \)-globulin fraction of plasma contains the immunoglobulins, a group of glycoproteins with great physiological importance, as all the immunoglobulins have antibody activity.
There are several immunoglobulins present in the serum and their complexities warrant summarizing their nomenclature in a table (117) given in Table 1. The protein structure of the immunoglobulins has been studied extensively (117-119) and a model of a typical immunoglobulin structure has been presented (117) in Figure 1.

Table 1. Nomenclature of Immunoglobulins

<table>
<thead>
<tr>
<th>Subunits</th>
<th>Light Chain</th>
<th>Heavy Chain</th>
<th>Immunoglobulin (Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td>k</td>
<td>$\gamma^a, \gamma^b, \gamma^c, \gamma^d$</td>
<td>$(k\gamma^a)_2 = \text{IgG}_a$, etc.</td>
<td>$\lambda\gamma^a$</td>
</tr>
<tr>
<td>$\lambda$</td>
<td>$\alpha^a, \alpha^b$</td>
<td>$(k\alpha^a)_2 = \text{IgA}_a$, etc.</td>
<td>$\lambda\alpha^a$</td>
</tr>
<tr>
<td>$\mu$</td>
<td>$\mu_\alpha$</td>
<td>$(k\mu)_10 = \text{IgM}$</td>
<td>$\lambda\mu$</td>
</tr>
<tr>
<td>$\delta$</td>
<td>$\delta^a$</td>
<td>$(k\delta^a)_2 = \text{IgD}$</td>
<td>$\lambda\delta$</td>
</tr>
<tr>
<td>$\epsilon$</td>
<td>$\epsilon^a$</td>
<td>$(k\epsilon^a)_2 = \text{IgE}$</td>
<td>$\lambda\epsilon^a$</td>
</tr>
</tbody>
</table>

1^Taken from reference (95).

It has been established (95) that immunoglobulins consist of non-identical subunits, light and heavy chains, of several classes. Each chain is composed of two sequences
Figure 1. A schematic representation of an immunoglobulin molecule. The variable portion and constant portion are labeled V and C, respectively. It is not known whether V is the same length in H and L chains.¹

¹Taken from reference (96).
of amino acids linked together. There is a variable sequence, which determines antibody specificity, and a constant sequence, which is characteristic of the species and determines the class of immunoglobulin as well as polymorphism within the class. Light and heavy chains are linked together by disulphide bonds to form the immunoglobulin. They may also occur in the form of a dimer (Figure 2), or may be of higher polymers. The carbohydrate may be attached at one or more points in the chain.

It appears that the light chains of the immunoglobulin molecule do not contain any carbohydrate (120,121) and that the carbohydrate of the molecule is associated with the heavy chain. In the case of human 7S γ-globulin (IgG) Clamp and Putnam (120) have shown that there are two oligosaccharides per molecule. The carbohydrate part of each molecule is formed by eleven hexose, eleven hexosamine, two fucose and one sialic acid residues. The two units are possibly not identical as can be seen by the odd number of residues. There are probably three acetoamido sugar molecules present as non-reducing end groups. Fucose is terminal.

Somewhat similar to IgG is 19S γ-globulin (IgM). The light chains of the two immunoglobulins are similar both in amino acid composition and lack of carbohydrate (122). The
heavy chains of the two immunoglobulins are quite distinct. The IgG contains two to three per cent carbohydrate (123) while that of IgM contains 12 per cent carbohydrate (124). The IgM has more hexose (6% vs 1%), more fucose (0.74% vs 0.29%), more hexosamine (3.31% vs 1.14%), and more sialic acid (2.01% vs 0.22%) than the IgG (123,124).

The blood plasma also contains several hormones which contain carbohydrate (125) including the gonadotropic hormones, intestinal cell stimulating hormones, leutenizing hormone, follicle stimulating hormone, choronic gonadotropin, and in horses, pregnant mare serum gonadotropin.

Enzymes present in blood plasma have also been shown to contain carbohydrate. These include cholinesterase and ribonuclease B (125).

The blood group specific substances have also been shown to contain carbohydrate (51).

8. Fetuin

One other animal glycoprotein which has been studied to a great extent is fetuin. Isolation of fetuin was originally reported by Pedersen (78). Spiro (24) isolated fetuin from fetal calf serum in high purity. He reported that the molecule, of molecular weight 48,400, consisted of 74%
polypeptide, with the remainder being carbohydrate composed of galactose, mannose, glucosamine, galactosamine and sialic acids. The sialic acids were primarily present as the N-acetyl form with about 7% in the N-glycolyl form. The majority of the sialic acids were in the terminal position of the heterosaccharide residue. No fucose was found in the molecule.

Spiro (126) has further reported that the fetuin molecule contains three polysaccharide units, each of molecular weight 3,500. The heterosaccharide units were likely branched, each containing four peripherally located sialic acids. The amino acids in the area of the carbohydrate-peptide linkage were aspartic acid, alanine, serine and proline.

E. BIOSYNTHESIS OF GLYCOPROTEINS

Glycoprotein biosynthesis involves the synthesis of both peptide and carbohydrate units. It is accepted that the polypeptide unit is synthesized by normal mechanisms of protein synthesis. Several workers have presented evidence to show that addition of carbohydrate only occurs after the polypeptide chain has been synthesized (127,136). The exact mechanism of the attachment of the carbohydrate remains unknown. Roseman (137) has proposed three possible answers.
Firstly, the oligosaccharide chains may be synthesized in a step-wise manner, with each residue added sequentially to the end of the chain. This view has been supported by others (55, 56). Secondly, the entire carbohydrate unit may be synthesized at the nucleotide level and then incorporated into the glycoprotein. Finally, the mechanism of synthesis may be a combination of the above two processes, whereby, the inner monosaccharides are first incorporated, followed by the addition of short oligosaccharides to give the final molecule. The work of Li, Li and Shetlar (138) has provided some support for this hypothesis.

The major site of biosynthesis of blood plasma glycoproteins is the liver (127, 128, 131-136, 139-142) and more specifically the microsomal membrane of the liver (143-149). Sarcione, Bohne and Leahy (139) have proposed that incorporation of carbohydrate occurs in the membranes of the endoplasmic reticulum after the peptide has been synthesized and released by the ribosome. This observation would support the first theory of Roseman. Bekesi and Winzler (150) have reported that removal of hepatic tissue in the rat had little effect on the incorporation of labeled fucose into other tissues. They concluded that although the liver is the primary site of glycoprotein synthesis, there is extrahepatic
synthesis of glycoprotein and that in fact, extrahepatic tissues do contribute to circulating glycoprotein.

Sutherland and Jackson (151) have found that the kidney plays a role in the control of glycoprotein synthesis by the liver, possibly through release of an inhibitor. The biosynthesis of the various carbohydrate constituents of glycoproteins may be summarized in a diagram (Figure 2) (152).

1. Hexose

The hexoses, galactose and mannose are derived from glucose as is shown in Figure 2. The hexoses once formed are then activated by conversion to the UDP derivatives.

The incorporation of hexoses in glycoproteins is catalyzed by enzymes, present in the microsomes, which are specific for the particular molecule. This is in accordance with the "one enzyme-one linkage" concept of Hagopian and Eyler (56). They suggested that the peptide chains are synthesized in the usual manner in the polysomes of the rough endoplasmic reticulum. Following this, they migrate to the smooth membrane fraction which contains a multienzyme group of glycosidic transferases, each being highly specific for the synthesis of a particular glycosidic linkage. The
Figure 2.1 Metabolism of carbohydrate constituents of glycoprotein

1Taken from reference (152).
final structure of the carbohydrate is dictated by the specificity of the transferase for the sugar previously attached. Sarcione (130) has shown that the site of incorporation of hexoses is indeed the membranous fraction of the microsome. Hagopian et al. (55,153) and Spiro and Spiro (154) have isolated glycoprotein-galactosyl transferases catalyzing the transfer of galactose from UDP-galactose to a suitable glycoprotein acceptor.

2. Hexosamine

The pathway of synthesis of the hexosamines and their N-acetyl derivatives is shown in Figure 2. UDP-derivatives are formed and the hexosamine is transferred to the peptide by a specific transferase enzyme (55,56,153,154).

Kornfeld and Noll (155) have studied the synthesis of glucosamine-1-\(^{14}\)C from fructose-\(^{14}\)C in rabbit erythrocytes. The initial and rate limiting enzyme in the active erythrocytes was found to be L-glutamine-D-fructose-6-phosphate-amino-transferase. Tesoriere, Macaione and Nicotra (156) also found that glutamine was necessary for the synthesis of glucosamine and glucosamine-6-phosphate in liver homogenates.

Gan, Trujillo and Chaikoff (157) have shown that the label from D-glucosamine-1-\(^{14}\)C remains in the C-1 group.
There is relatively little breaking of the hexose chain.

3. Sialic Acids

The synthesis of neuraminic acid may be seen from Figure 2. The biosynthesis of N-acetylmuramic acid from fructose-6-phosphate is indicated (158). The N-acetyl form may then be converted to the N-glycolyl form. Conversion requires the presence of N-acetyl neuraminate and N-acetyl hydroxylase. In addition, acetylmuraminic acid transferase and glycolymuraminic acid transferase must be present (159).

The neuraminic acids, once formed, are activated by conversion to their CMP (cytidine monophosphate) derivatives. The necessary CMP-neuraminic acid synthetase has been isolated (44,158). The CMP-neuraminic acids are then transferred to the glycoprotein by specific glycosyltransferases (44,158). Transfer is usually to terminal N-acetyl galactosamine (44).

4. Fucose

The biosynthesis of fucose from glucose is indicated and it appears that synthesis occurs through mannose (160, 161). Ginsberg (162) has shown that conversion of
Guanosinediphosphate-mannose to fucose involves at least two individual reactions:

\[
\text{GDP-D-mannose} \rightarrow \text{GDP-4-keto-6-deoxy-D-mannose} \rightarrow \text{GDP-L-fucose.}
\]

The primary pathway of synthesis is through GDP-mannose. Coffey, Miller and Sellinger (163) have suggested that there is also a mechanism whereby L-fucose may be directly activated without conversion to GDP-D-mannose. Ishihara, Massaro and Heath (164) have in fact isolated an L-fucose kinase which catalyses the reaction:

\[
\text{L-fucose} + \text{ATP} \rightarrow \text{\(\beta\)-L-fucose-1-phosphate} + \text{ADP}.
\]

Furthermore, Ishihara and Heath (165) have isolated a GDP-L-fucose pyrophosphorylase. The GDP-L-fucose thus formed is probably acted on by a fucosyltransferase resulting in incorporation into glycoprotein.

F. ANALYTICAL METHODS

1. Protein

The amount of protein present in a sample of serum or of glycoprotein has been determined by two methods. Protein may be determined by the biuret reaction (166,167) or by the Lowery-Folin method (168), or by modifications of these methods (169,170). These methods were standardized by
the determination of amino nitrogen by the Kjeldahl method (171,172).

Quantitation of amino acids may be performed by the automatic amino acid analyzer, based on the technique of Spackman, Stein and Moore (173), or by gas chromatography (174,175,176).

2. Hexoses

Neutral hexoses may be determined by the Anthrone reaction (177,178,179), by the Orcinol reaction (180), by the Somogyi Nelson method (181) and by others (182).

The hexoses may also be determined by ultraviolet absorption following treatment with acid (183).

Individual hexoses may be determined by paper chromatography (184-188), column chromatography (24,189,190), thin layer chromatography (188,191,192) and gas chromatography (193,194).

3. Hexosamine

Most colorimetric methods for the estimation of hexosamine are based on the method of Elson and Morgan (195). Modifications have been proposed by Rondle and Morgan (176) and others (182,197-203). Other methods have been proposed (204,205).
Estimation of hexosamines by these methods is usually preceded by a separation of the hexosamines from interfering substances (206-210).

Hexosamines may also be separated and quantitated by paper chromatography (187,188), by thin layer chromatography (191,211), by gas-liquid chromatography (212-214) and by ion exchange chromatography (215,216).

4. Sialic Acid

Sialic acid has been determined colorimetrically by the thiobarbituric acid method (217-219), the orcinol (220, 221) and resorcinol (219,222) methods, the direct Ehrlich method (223,224) and others (182).

Sialic acid may also be separated and quantitated by paper and thin layer chromatography (188) and by gas-liquid chromatography (224).

5. Fucose

Fucose has been determined by the cystein-H₂SO₄ method of Dische and Shettles (225) and the thioglycolic acid method of Gibbons (226). Recently Tsignos and Muir (227) have described a modification of the method of Yemme and Willis (228) whereby fucose may be estimated without interference from
hexoses, hexuronic acids, hexosamines and sialic acids.

Fucose has also be determined by thin layer chromatography (211).

6. Electrophoresis

Serum and plasma proteins have been separated by several different electrophoretic techniques, including moving boundary free electrophoresis (229-232) and zone electrophoresis in paper (71,73,233), cellulose acetate (234,235), agar (236-238), polyacrylamide gel (72,239-243), starch gel (244) and polyvinylchloride (245). Immunoelectrophoresis has also been employed (246), as has isoelectric focusing (247).

Tiselius (248) studied the electrophoretic distribution of carbohydrate by moving boundary free electrophoresis. The electrophoretic distribution of carbohydrate following zone electrophoresis in paper has been studied extensively (71,249-267).

Cellulose acetate membranes have also been employed (234,268,269).

Agar electrophoresis (270-272) has been employed for both analytical and preparative work on glycoproteins.

Polyvinylchloride electrophoresis has been employed for the separation of serum proteins, followed by elution and
determination of carbohydrates (273-277).

Electrophoretic distribution of carbohydrate has also been determined by polyacrylamide gel electrophoresis (278) and starch gel electrophoresis (279, 280).
II. ANALYTICAL TECHNIQUES

A. COLLECTION AND TREATMENT OF BLOOD

1. Preparation of Serum

Blood was collected in screw cap test tubes and allowed to clot at room temperature until serum was observed to separate from the clot, usually two to three hours. The clear yellow serum was then decanted and was centrifuged twice. If serum was not to be immediately used for analysis or electrophoresis it was frozen.

2. Preparation of Plasma

Blood was collected in 0.1 volumes of 5% EDTA (tetra-sodium ethylene diamine tetra acetic acid) in 0.9% aqueous sodium chloride. Care was taken to make sure that the blood was well mixed with the EDTA solution. The plasma was centrifuged to remove blood cells. The plasma was processed immediately for the fractional precipitation of the proteins with ammonium sulphate.

B. HYDROLYSIS PROCEDURES FOR THE RELEASE OF CHARACTERISTIC GLYCOPROTEIN CONSTITUENTS

1. Preparation of Dowex Resin (Dowex 50W-X8, Cation Exchange Resin 200-400 Mesh)
The resin was placed in a large beaker, and was suspended in water. The resin was allowed to settle for 10 - 15 minutes, at which time the supernatant was removed. This process was repeated until most of the fines had been removed.

The resin was transferred to a large fritted glass funnel and was washed with two portions of 2 N sodium hydroxide. The resin was then washed with distilled water until the washings were neutral to litmus paper. The resin was then washed with two portions of 2 N hydrochloric acid, followed by water washes until a negative chloride ion test (silver nitrate) was obtained. The above washing with sodium hydroxide and hydrochloric acid was repeated. The resin was then washed with two portions of 0.075 N hydrochloric acid. The resin was then allowed to drain and was suspended in 0.075 N hydrochloric acid (1:2 w/v). Resin was stored in a closed container at room temperature.

If after standing for a length of time the supernatant became orange in colour, the resin was not used for hydrolysis, but was regenerated according to the above procedure.

2. *Hydrolysis Procedures*
a. For the Release of Hexosamine and Hexoses

One ml of serum or 10 - 20 mg of isolated glycoprotein was placed in a hydrolysis tube (Pyrex test tube 13 x 100 mm) with 4 ml of the Dowex suspension. The tube was then sealed. Hydrolysis was carried out in an oven with a rotating vertical tube holder (281) at 100°C for 24 - 36 hours.

b. For the Release of Fucose

The conditions of hydrolysis for the release of fucose were the same as for hexosamine, except that the length of hydrolysis was 12 hours.

c. For the Release of Sialic Acid

0.1 ml of serum or 1 - 2 mg of isolated fraction were placed in a hydrolysis tube with 1 ml of 0.1 N sulphuric acid. The tube was sealed. Hydrolysis was carried out in an oven with a rotating vertical tube holder at 80°C for 60 minutes.

3. Chromatography of Products of Resin Hydrolysis

The hexosamines, hexoses and fucose were eluted from the Dowex resin. The contents of the hydrolysis tubes were transferred with the aid of 10 ml of distilled water to a
glass column (1 x 30 cm) with a fritted disc. The eluates (water eluate A) were collected in 25 ml beakers. Twenty-five ml volumetric flasks were then placed under the columns. Elution was continued with 10 ml of 2 N hydrochloric acid in three portions, followed by 5 ml of distilled water to wash out remaining acid. This was the acid eluate, which contained the hexosamines. The volumetric flasks were changed and the water eluate A was passed through the column. The column was washed with 11 ml water in three portions. The flasks were then replaced with those containing the acid eluates. Any remaining hexosamine was eluted from the column with 10 ml of 2 N hydrochloric acid. Flasks were made up to volume with distilled water.

C. DETERMINATION OF CHARACTERISTIC COMPONENTS OF GLYCOPROTEIN

1. Hexosamine

Hexosamine was determined by the modified Elson and Morgan Reaction of Anastassiadis, Maw and Common (199). An aliquot of hydrolysate sufficient to give a suitable absorbance was pipetted into 10 ml volumetric flask with ground glass stoppers. A solution of 4% acetylacetone (2.4 pentane-dione) in 0.75 M sodium carbonate was prepared immediately
before use. Two ml of acetylacetone solution was added to the flasks. The flasks were stoppered tightly, shaken and placed in a wire basket. They were heated in a boiling water bath for one hour, after which they were cooled under running tap water.

The flasks were opened and 5 ml of absolute ethanol was added. The flasks were shaken and two milliliters of Ehrlich's Reagent (0.8 gk of P-dimethyl-aminobenzaldehyde dissolved in 30 ml absolute ethanol, and 30 ml concentrated hydrochloric acid was added slowly with shaking so as to prevent excessive foaming.

The flasks were made up to volume with absolute alcohol and the absorbance (A 530) of the solution, at 530 mυ, was determined in a spectrophotometer (Bausch and Lamb Spectronic 20).

A blank, and glucosamine hydrochloride standards were run simultaneously. A standard curve (Fig. 3) was prepared and the average concentration:absorbance ratio, K, was found. The amount of hexosamine (H) was determined by the equation,

\[ H = A_{530} \times D.F. \times K \times 0.8303 \]

where D.F. = dilution factor.

2. Hexose

Hexoses were determined by the anthrone reaction
according to the method of Fairbairn (177). Anthrone reagent was prepared by adding one gram of anthrone to sulphuric acid, made by diluting 760 ml of concentrated acid to one liter. An aliquot of hydrolysate, usually 2 ml was pipetted into a test tube (16 x 150 mm). Ten ml of anthrone reagent was added. The contents were mixed, and heated in a boiling water bath for 12 minutes. The tubes were cooled under tap water, mixed, and the absorbance of the solution measured at 620 m\(\mu\) in a spectrophotometer. A standard curve was prepared (Fig. 4).

3. Sialic Acid

Sialic acid was determined by the thiobarbituric acid method of Warren (217). An aliquot of hydrolyzate was placed in a test tube (13 x 150 mm). To this, 0.1 ml of 0.2 M sodium metaperiodate in 9 M phosphoric acid was added. The tube was shaken to ensure complete mixing. The tubes were allowed to stand for 20 minutes at room temperature. One ml of a 10% solution of sodium arsenite in a 0.5 M solution of sodium sulphate in 0.1 N sulphuric acid was rapidly added. The contents were shaken until a yellow brown colour which formed had disappeared. Following this, 3 ml of a 0.6% thiobarbituric acid in 0.5 M sodium sulphate solution was added. The tubes were again shaken and were loosely capped.
They were heated in a boiling water bath for 15 minutes. Following heating the tubes were cooled to room temperature in tap water, and 3.5 ml of cyclohexanone was added. The tubes were shaken then centrifuged. The absorbance of the cyclohexanone layer was determined at 549 m\(\upmu\). A standard curve was prepared (Fig. 5).

4. Fucose

Fucose was determined by the method of Tsiganos and Muir (227). Three ml of anthrone reagent was frozen in a test tube (12 x 150 mm, glass stoppered) using liquid air. One ml of hydrolysate or standard fucose solution was layered on top of the frozen reagent. The tubes were immersed in an ice bath. The tubes were shaken gently as the anthrone reagent melted and vigorously after melting so as to obtain a gradual but complete mixing. The tubes were then placed in a water bath at 40\(^{\circ}\)C for exactly 30 minutes. They were then cooled under tap water and allowed to stand for 20 minutes in the dark. The absorbance at 640 m\(\upmu\) was determined in a spectrophotometer. Concentration of sample was determined by consulting a standard curve (Fig. 6) and applying the appropriate dilution factor.

5. Protein Determination
a. Biuret Reaction

Protein of whole sera was determined by the biuret reaction of Weichselbaum (166). An aliquot of serum (0.1 ml), 4.9 ml of 0.85% sodium chloride and 5 ml of Biuret reagent (45 gm of sodium potassium tartrate, 15 gm copper sulphate and 5 gm of potassium iodide dissolved in one liter of 0.2 N sodium hydroxide) were pipetted into a glass stoppered test tube (16 x 150 mm) and were mixed. The tubes were placed in a water bath at 28 - 32°C for 30 minutes. The contents were mixed and their absorbance at 555 m\u00b5 was determined. The method was standardized by use of the Micro-Kjeldahl method (172) (Fig. 7).

b. Folin-Ciocalteau Method

Protein concentration was estimated by the Folin-Ciocalteau method of Lowry (168). An aliquot of sample was placed in a test tube and 3 ml of a fresh 50:1 mixture of 2% sodium carbonate and 0.5% copper sulphate in 1% sodium potassium tartrate was added. The solution was mixed well and allowed to stand at room temperature for 10 minutes. Then 0.3 ml of Folin Phenol Reagent (Fisher Scientific, diluted 2:1 to be 1 N) was rapidly added. The tubes were immediately shaken. After standing at room temperature for 30 minutes the absorbance at 500 m\u00b5 was determined. A calibration curve was prepared (Fig. 8).
Figure 3. Standard curve for the determination of hexosamine by the method of Anastassiadis, Maw and Common.
Figure 4. Standard curve for the determination of hexoses by the method of Fairbairn.
Figure 5. Standard curve for the determination of sialic acid by the thiobarbituric acid method of Warren.
Figure 6. Standard curve for the determination of fucose by the method of Tsiganos and Muir.
Figure 7. Standard curve for the determination of protein by the method of Weichselbaum.
Figure 8. Standard curve for the determination of protein by the Folin-Ciocalteau method.
D. ELECTROPHORETIC TECHNIQUES

1. Paper Electrophoresis

a. Electrophoretic Procedure

The determination of the distribution of protein and hexosamine was performed by the method as described by Gaunce and Anastassiadis (71). Whatman 3 MM filter paper was cut into strips 17.5 cm x 30.5 cm. A strip was placed in the electrophoresis chamber (horizontal, Matthew cell Model PE-103; Matthew Laboratories, Yonkers, N.Y.) and allowed to equilibrate with buffer (Veronal buffer pH 8.6, μ = 0.05) for at least one half-hour. After the strip was wet with buffer, the sample was applied 9 cm from the cathodic end. The serum, 0.2 - 0.3 ml was applied from a 0.5 ml graduated pipette in a straight band across the filter paper strip. Electrophoresis was conducted in a cold room at 4°C for 24 hours at 4 ma.

b. Protein Stain

Following the electrophoretic run, the strip was removed from the chamber and allowed to dry at room temperature. The dry electropherogram was then stained with a saturated solution of amido Schwartz stain (Amido Schwartz 10B, Chromagellschaft, Stuttgart, Germany) in acetone:glacial
acetic acid (9:1), for twenty minutes. The strip was then washed repeatedly in a solution of methanol:water:1 N hydrochloric acid (90:10:1) by volume. The electropherogram was finally rinsed in methanol and allowed to air dry.

The distribution of protein on the electropherogram was determined by scanning a portion of the stained strip in a recording densitometer (Beckman Analytrol, with 500 mμ filters and a B-2-cam.). The per cent distribution of protein in each zone was determined from the scanning.

c. Hexosamine Distribution

Following the determination of the protein distribution, the entire electropherogram was cut into strips according to the pattern revealed by the protein stain. Each strip was then cut into small portions and sealed in a test tube (Pyrex 13 x 100 mm) with 2 N hydrochloric acid. Hydrochloric acid was used in the amount of 1.5 ml of acid for each 0.1 gm of paper. Hydrolysis was carried out in a boiling water bath for six hours.

Following hydrolysis, the tubes were centrifuged, opened, and decanted into 25 ml beakers. To each tube 0.75 ml of water per 0.1 gm of paper was added. The tubes were shaken, centrifuged and the water was decanted. This washing step was repeated twice more. The acid and water washings were then evaporated to dryness, over sodium hydroxide,
in a vacuum desiccator.

The dried eluate was redissolved in 5 ml water and was added to a fritted glass column (1 x 30 cm) containing 5 cm of Dowex resin. The column was then washed with two 5 ml portions of water, the eluate being collected in 25 ml beakers. The column was then washed three times using a total of 15 ml of 2 N HCl, to elute the hexosamines. The acid eluate, also collected in beakers, was taken to dryness over sodium hydroxide in a vacuum desiccator. The dry sample was then analyzed for hexosamine (section II,C,1). The amount of hexosamine in each zone was then determined.

2. Cellulose Acetate Electrophoresis

a. Electrophoretic Procedure

Serum proteins were separated on cellulose acetate strips (Sepharose III Cellulose polyacetate strips, Cat. No. 51003, 1" x 6 3/4". Gelman Instrument Company, Ann Arbor, Michigan), according to the manufacturer's instructions (282). Electrophoresis was performed in a Shandon Electrophoresis Apparatus using veronal buffer (pH 8.6, $\mu = 0.01$).

The cellulose acetate strips were carefully floated on the surface of fresh buffer. Only after the entire strip had become moist was it submerged. Once wet in this manner
the strip was immediately used. The strip was removed from the buffer and was blotted gently between two sheets of filter paper. While the strip was on the filter paper about 5 - 10 \( \mu l \) of serum was applied using a dual wire application (Spinco sample striper No. 300-805, Specialized Instruments Corporation, Belmont, California). The strip was then immediately transferred to the chamber, care being taken that the strip remained horizontal.

When all the strips had been placed in the chamber in this manner, the power was applied (100 V at 4-5 ma per strip) for one to one and a half hours. The strips were removed and dried in an oven at 80°C for 30 minutes. The strips were then placed in 95% ethanol for 10 - 15 minutes, after which they were stained for protein or glycoprotein.

b. Protein Stain

Cellulose Acetate strips were transferred from the 95% ethanol, to stain solution (0.5% Amido black in 9:1 methanol:acetic acid) where they were left for 5 minutes. The strips were then destained in 9:1 methanol:acetic acid until the background was white. Strips were usually scanned in the Photovolt recording densitometer and photographed prior to clearing.
c. Glycoprotein Stain

Cellulose acetate electropherograms were stained for glycoprotein by a periodic acid-schiff stain (234).

Strips were removed from the 95% ethanol and were placed in periodic acid solution (2.5 gm periodic acid in 50 mls of 0.2 M sodium acetate diluted to 500 mls) which was prepared immediately before use, for 8 to 10 minutes. The strips were then rinsed in 0.001 N hydrochloric acid and were then transferred to potassium iodide solution prepared immediately before use by adding 10 ml of 0.1 N HCl and one drop of saturated thiosulphate solution to 400 ml of 10% KI. The strips were left in the potassium iodide solution until the yellow colour, which formed immediately, had disappeared. The strips were again rinsed in 0.001 N hydrochloric acid. They were then stained for 20 minutes in Schiff reagent: the Schiff's reagent was prepared by dissolving 2 g fuchsin-base (Allied Chemical, Special for Flagella Staining) in 400 ml of distilled water, to which 10 ml 2 N hydrochloric acid and 4 g potassium metabisulphite were added. Then 10 ml 2 N hydrochloric acid and 4 g activated carbon were added, and the mixture was filtered.

The stained strips were placed in three successive baths for 15 minutes each, of 0.1 N nitric acid. The strips
were immediately scanned and photographed as they faded noticeably within a few days.

d. Clearing of Cellulose Acetate Electropherograms

Cellulose acetate electropherograms were cleared, i.e. made transparent, following dehydration in two rinses of absolute methanol. After a rinse in 9:1 methanol, acetic acid solution, the strips were placed on a glass plate, care being taken that all air bubbles and wrinkles were excluded. As the strips dried, they became transparent.

3. Agar Electrophoresis

Agar electrophoresis was performed according to the method of B.R. Das (271). The electrophoretic trough was constructed with the modification as proposed by A.P. Gaunce (283). The trough was made of plexiglass and was 28 x 7.6 x 2 cm with slits in the end piece.

A one per cent agar solution was prepared by autoclaving 1.2 gm of Agar Nobel (Difco Laboratories, Detroit) in 120 ml of water at 15 lb/in² for 15 minutes. The bottom of the trough was coated with about 2 - 3 mm of the agar solution and Whatmann 3 MM filter paper wicks were inserted through the slits. The wicks were allowed to soak up agar
solution so that when the agar gelled, the slits were sealed. The remaining agar solution was mixed with an equal volume of veronal buffer (pH 8.6, \(\mu = 0.1\)) and was poured into the level trough. At the same time, a sample slot was prepared by having a piece of plexiglass 5 cm x 0.25 cm supported in the agar solution. After the agar gelled, the plexiglass form was removed, leaving a sample well. The sample (1 ml of serum) was mixed with one ml of the veronal buffer (pH 8.6, \(\mu = 0.1\)) and was placed in the sample slot, with one drop of a tracking dye (1% bromophenol blue). The wicks were dipped into electrode tanks holding veronal buffer (pH 8.6, \(\mu = 0.05\)). Electrophoresis was conducted at 250 to 300 V (10 mA) for about 20 hours.

After completion of the run the gel was cut into segments according to the pattern revealed by a test print. The test print was made by allowing a piece of filter paper to soak up solution for a few seconds and then staining for protein. Each segment was placed in a test tube, frozen and then thawed to extract the liquid.

Aliquots of the eluate were analyzed for hexosamine (Sect. II,C,1) and for sialic acid (Sect. II,C,3) in order to determine the amounts of each component in the various zones.
4. Acrylamide Gel Electrophoresis

a. Electrophoretic Procedure

Horizontal acrylamide gel electrophoresis (239) was conducted in plexiglass cells with a gel size of 17.5 x 9 x 0.3 cm and containing a cooling plate. A 7% solution of Cyanogum 41 in Tris-borate-EDTA buffer (20.16 g tris (hydroxymethyl) amino methane 2.62 g disodium ethylene-diamine-tetra-acetic acid and 1.57 g boric acid per 2,000 ml distilled water) was prepared. To 100 ml of this solution, 0.1 g ammonium persulphate was added, then 0.2 ml of dimethyl-amino-propionitrile was added. The gel solution was then poured into the leveled plexiglass form and it was covered with a sheet of plexiglass held down with lead weights. Care was taken to prevent the trapping of air under the plexiglass cover. After the acrylamide had gelled the cover plate was removed.

The plexiglass form was then supported on buffer troughs and Whatman 3 MM filter paper wicks were used to connect the gel to buffer in the troughs. Samples were applied on small pieces of filter paper inserted into a slit cut across the width of the gel about 3 cm from the cathodic end. The gel was covered with a sheet of Saran Wrap. A voltage of 300 V (20 ma) was applied for seven hours. The
gel was then removed from the vessel and stained.

b. Protein Stain

The gel was fixed by washing in a bath of 15% acetic acid for 15 minutes. The gel was then transferred to a solution of Amido Schwartz 10B (1 gm in 150 ml methanol, 700 ml glacial acetic acid, and water to 2 liters), for 15 - 20 minutes. The background was destained with 15% acetic acid.

c. Glycoprotein Stain

Carbohydrate was stained by a modified Schiff's stain (270). The gel was fixed for 15 minutes in 15% HAc, then was transferred to a 0.5% potassium periodate solution for 45 minutes. The gel was then rinsed in distilled water and transferred to the fuchsin-sulphite solution for 15 minutes.

The fuchsin-sulphite solution was prepared by dissolving 1 gm basic fuchsin (Allied Chemical, special forFlagella stain) in 1,000 ml of water, and adding 5.5 gm of potassium bisulphite (meta) and 10 cc of conc. HCl. The solution was allowed to stand until it was colourless or clear yellow.

Following the fuchsin sulphite stain the gel was rinsed in distilled water then transferred to a 0.4%
potassium metabisulphite solution. The gel was washed repeatedly in this solution until the background was destained.

E. ISOLATION OF PLASMA GLYCOPROTEIN

1. **Fractional Precipitation of Plasma Proteins**

Bovine plasma was fractionated according to the procedure of Bezkorovainy and Doherty (85). Ammonium sulphate (300 gm per liter of plasma) was added to the plasma and when it was dissolved the pH of the mixture was adjusted to 4.7. The mixture was allowed to stand for 16 hours at 4°C, at which time a precipitate, P-1, was obtained by centrifugation. The supernatant was readjusted to pH 3.7 and allowed to stand 16 hours at 4°C. The precipitate, P-2, was obtained by centrifugation. Ammonium sulphate (100 gm per liter of plasma) was added and the pH was adjusted to 3.7. After standing a further 16 hours at 4°C precipitate P-3 was obtained by centrifugation. The final precipitate was obtained by adding ammonium sulphate (100 gm per liter) to the supernatant, allowing it to stand for 16 hours at 4°C and centrifuging. The precipitates were dissolved in distilled water, dialyzed against distilled water for 24 to 48 hours and freeze dried.
2. **Carboxymethyl Cellulose Chromatography of Plasma Fractions**

a. **Treatment of Carboxymethyl Cellulose Resin**

Carboxymethyl cellulose (Brown Company, Berlin, New Hampshire, type 20, exchange capacity of 0.72 meq. per gm.) was suspended in distilled water and allowed to settle for 20 minutes. The supernatant including unsettled fines, was removed by suction. The resin was then suspended in 0.2 N hydrochloric acid, allowed to settle and the supernatant removed. The resin was then washed with distilled water until a negative chloride ion test (silver nitrate test) of the washings was obtained. In a similar manner, the resin was treated in succession with 0.2 N sodium hydroxide, water, hydrochloric acid, water, sodium hydroxide, water, hydrochloric acid, and finally water. The resin was stored at 4°C suspended in water. The resin was used soon after the treatment.

b. **Chromatography on Column of CM-Cellulose**

A sufficient amount of resin to give a 20 to 30 cm column was suspended in distilled water and added to the column (pyrex glass tube 3.5 cm x 60 cm). The total charge of resin was added at once to avoid layering in the column.
The column was then washed with 100 ml of distilled water and 100 ml of starting buffer (acetate buffer pH 4.1), after which it was ready for application of the sample.

Up to 5 g of fraction (P-1, P-2, P-3 or P-4) were dissolved in a minimal amount of starting buffer. The starting buffer already in the column was allowed to drain down level with the resin. The sample solution was then carefully layered on top of the column and allowed to drain in. Then about 50 ml of the buffer was layered on and allowed to drain in. The level of buffer was then established and a constant head device fitted. Chromatography was allowed to proceed, at a buffer flow rate of 40 to 50 ml per hour. Buffer was collected in 15 ml volumes in an automatic fraction collector. When the fraction was observed to come off the column, as determined either by an ultraviolet flow monitor (Buchler Instrument Co., Uviscan) or by reading of optical density of tube at 280 m\textmu; in a Beckman DU spectrophotometer, the buffer was allowed to run down until level with the resin. Fifty ml of buffer II (acetate buffer pH 5.0) was run into the column. The hydrostatic head was then re-established and chromatography continued. In a similar manner, the column was washed with buffer III (phosphate buffer pH 7.0). In some cases a fourth buffer, buffer IV
(phosphate buffer pH 8.0) was used. The contents of tubes containing a single fraction were pooled. The pooled fractions were dialyzed against distilled water and freeze dried.

F. LIQUID SCINTILLATION COUNTING

Quantitative estimates of the amount of $^{14}$C present in samples were made using a Packard 3003 TriCarb Liquid Scintillation Spectrometer. Counting was in the green channel with settings as follows:

- Green channel $C=50$  $D=1000$  gain 9% window $C-D$
- Blue channel $E=350$  $F=1000$  gain 2% window $E-$

Quenching corrections were made by the external standard method (284) (Fig. 9).

1. Sample Preparation for Liquid Scintillation Counting

a. Serum

To 0.1 ml of serum, 1.5 ml of absolute alcohol was added. The tubes were centrifuged and the alcoholic supernatant was decanted into scintillation vials (aluminum lined screw-cap, glass vials). The alcohol was evaporated. To the vial, 15 ml of scintillation fluid I (0.01 per cent POPOP and 0.4 per cent PPO in toluene) was added.

The protein precipitate was dissolved in two 1 ml
Figure 9. Standard curve for the determination of counting efficiency by the automatic external standard method.
portions of hyamine hydroxide (Packard Inst. Co., 2200 Womenville Road, Downes Grove, Illinois, U.S.A.). The hyamine solutions were placed in counting vials. To the vials 15 ml of scintillation fluid I was added.

b. Plasma and Protein Fractions

Samples of plasma or protein fraction were prepared for counting according to Mahin and Lofberg (285). 0.1 - 0.2 ml of plasma or 1 - 2 mg of fraction were placed in a counting vial. To this an equal volume of 60 per cent perchloric acid was added (0.2 ml in the case of protein fraction). The sample was well mixed with the acid. Twice the volume of 30 per cent hydrogen peroxide was added. The contents were again well mixed. The caps were then screwed on tightly. The vials were then placed in an oven at 70 - 80°C for 30 to 60 minutes.

Following digestion of the sample, 5 ml of cellosolve (2-Ethoxyethanol) and 10 ml of scintillation fluid II (0.6% PPO in toluene) were added.
III. RESULTS AND DISCUSSION

A. VARIABILITY IN THE HEXOSAMINE DETERMINATION

A study was made to examine the effect of size of reaction vessel and concentration of glucosamine on the variability of the results of the hexosamine determination (Sect. II, C, 1).

Standard solutions of 20, 40 and 60 μg of glucosamine-hydrochloride per ml were prepared. Analysis was carried out according to the procedure in 5 ml, 10 ml, 25 ml, 50 ml, and 100 ml ground glass stoppered volumetric flasks. Determinations were performed on 10 replicates in each size of flask and at each concentration level. The standard deviation of the observed absorbances were calculated. The mean values and standard deviations are given in Table 2. The standard deviations of absorbances of solutions in various sizes of vessels were compared by the F-test (Table 3). As can be seen by this treatment there would not appear to be any relation between size of reaction vessel and variability as determined by the standard deviation of absorbances.
Table 2. Absorbance and standard deviation of reaction solutions of glucosamine hydrochloride at various concentrations and in various sizes of reaction vessels for 10 replicates

<table>
<thead>
<tr>
<th>Vessel Size</th>
<th>Concentration</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 µg/ml</td>
<td>40 µg/ml</td>
<td>60 µg/ml</td>
<td></td>
</tr>
<tr>
<td>5 ml</td>
<td>0.167±0.017</td>
<td>0.347±0.027</td>
<td>0.498±0.040</td>
<td></td>
</tr>
<tr>
<td>10 ml</td>
<td>0.163±0.025</td>
<td>0.330±0.052</td>
<td>0.486±0.085</td>
<td></td>
</tr>
<tr>
<td>25 ml</td>
<td>0.141±0.026</td>
<td>0.344±0.047</td>
<td>0.506±0.039</td>
<td></td>
</tr>
<tr>
<td>50 ml</td>
<td>0.187±0.012</td>
<td>0.388±0.022*</td>
<td>0.525±0.024</td>
<td></td>
</tr>
<tr>
<td>100 ml</td>
<td>0.183±0.018</td>
<td>0.347±0.032</td>
<td>0.436±0.026</td>
<td></td>
</tr>
</tbody>
</table>

*Average of nine replicates.

B. COMPOSITION OF SERA IN CHARACTERISTIC GLYCOPROTEIN CONSTITUENTS

Samples of sera of mature female Holstein cows and mature female Landrace pigs were collected from animals of the Macdonald College Farm by Mr. W. Bogie. Samples of sera were collected from mature White Leghorn hens of the departmental flock.

All sera samples were analyzed for protein, hexose, hexosamine, sialic acid and fucose contents (sect. II,C,1,
Table 3. Statistical comparison of data of Table 2.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>20 µg/ml</th>
<th>40 µg/ml</th>
<th>60 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>V₁</td>
<td>V₂</td>
</tr>
<tr>
<td>5 and 10 ml</td>
<td>1.47</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>10 and 25 ml</td>
<td>1.04</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>25 and 50 ml</td>
<td>2.17</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>50 and 100 ml</td>
<td>1.50</td>
<td>9</td>
<td>9</td>
</tr>
</tbody>
</table>

All ratios were found to be non-significant at the 10% level.
2,3,4,5). Results of the analyses are given in Table 4. A statistical treatment by means of the "t" test of the data of Table 4 is presented in Table 5.

The level of hexosamine in porcine sera is slightly less than the levels in bovine and avian sera. Porcine serum contains twice as much hexose as does bovine and avian serum.

The greatest species variation is shown in the levels of sialic acid and fucose in the sera. Avian sera contain about half the amount of sialic acid than do the bovine or porcine sera.

In the case of fucose, porcine sera contain about twice as much as bovine and avian sera.

All the above variations are in the total content of the constituents in the serum. As all these constituents, with the exception of hexose, are protein bound, it is desirable to examine the variation in contents when expressed as a ratio with respect to protein. Because of the small amounts of constituents in relation to protein, the ratio times one hundred was calculated. The values of these ratios are given in Table 6. A statistical comparison by the "t" test of these ratios is presented in Table 7.

When the data are treated in this manner several
Table 4. Composition in protein, hexosamine, hexose, sialic acid and fucose of bovine, porcine and avian sera

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Bovine</th>
<th>Porcine</th>
<th>Avian</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of animals</td>
<td>Conc.</td>
<td>No. of animals</td>
</tr>
<tr>
<td>Protein (gm/100 ml)</td>
<td>5</td>
<td>9.7±0.2</td>
<td>5</td>
</tr>
<tr>
<td>Hexosamine (mg/100 ml)</td>
<td>5</td>
<td>94.5±3.4</td>
<td>5</td>
</tr>
<tr>
<td>Hexose (mg/100 ml)</td>
<td>5</td>
<td>123.0±5.6</td>
<td>5</td>
</tr>
<tr>
<td>Sialic Acid (mg/100 ml)</td>
<td>5</td>
<td>45.1±3.3</td>
<td>5</td>
</tr>
<tr>
<td>Fucose (mg/100 ml)</td>
<td>5</td>
<td>12.2±0.2</td>
<td>5</td>
</tr>
</tbody>
</table>
### Table 5. Statistical comparison of the data of Table 4. Probability levels at which the differences of concentration are significant

<table>
<thead>
<tr>
<th>Value Compared</th>
<th>Degrees of freedom</th>
<th>Value of t</th>
<th>Level of P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein Concentration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B/P</td>
<td>8</td>
<td>16.0</td>
<td>0.001</td>
</tr>
<tr>
<td>B/A</td>
<td>9</td>
<td>43.0</td>
<td>0.001</td>
</tr>
<tr>
<td>P/A</td>
<td>9</td>
<td>21.0</td>
<td>0.05</td>
</tr>
<tr>
<td>Hexose Concentration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B/P</td>
<td>8</td>
<td>3.3</td>
<td>0.001</td>
</tr>
<tr>
<td>B/A</td>
<td>9</td>
<td>0.6</td>
<td>0.05</td>
</tr>
<tr>
<td>P/A</td>
<td>9</td>
<td>20.3</td>
<td>0.001</td>
</tr>
<tr>
<td>Hexosamine Concentration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B/P</td>
<td>8</td>
<td>50.0</td>
<td>0.01</td>
</tr>
<tr>
<td>B/A</td>
<td>9</td>
<td>5.6</td>
<td>0.001</td>
</tr>
<tr>
<td>P/A</td>
<td>9</td>
<td>65.2</td>
<td>0.001</td>
</tr>
<tr>
<td>Sialic Acid Concentration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B/P</td>
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<td>2.6</td>
<td>0.01</td>
</tr>
<tr>
<td>B/A</td>
<td>9</td>
<td>9.0</td>
<td>0.001</td>
</tr>
<tr>
<td>P/A</td>
<td>9</td>
<td>0.7</td>
<td>0.001</td>
</tr>
<tr>
<td>Fucose Concentration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B/P</td>
<td>8</td>
<td>48.0</td>
<td>0.05</td>
</tr>
<tr>
<td>B/A</td>
<td>9</td>
<td>4.2</td>
<td>0.05</td>
</tr>
<tr>
<td>P/A</td>
<td>9</td>
<td>70.6</td>
<td>0.001</td>
</tr>
</tbody>
</table>

B/P = represents the comparison of concentration in bovine to concentration in porcine serum.
B/A = represents the comparison of concentration in bovine to concentration in avian serum.
P/A = represents the comparison of concentration in porcine to concentration in avian serum.

### Table 6. Ratio of constituent to protein concentration

<table>
<thead>
<tr>
<th>Ratio</th>
<th>Bovine</th>
<th>Porcine</th>
<th>Avian</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Hexosamine/Protein) x 100</td>
<td>0.95±0.06</td>
<td>1.23±0.05</td>
<td>1.61±0.08</td>
</tr>
<tr>
<td>(Hexose/Protein) x 100</td>
<td>1.37±0.15</td>
<td>3.32±0.25</td>
<td>1.91±0.22</td>
</tr>
<tr>
<td>(Sialic Acid/Protein) x 100</td>
<td>0.52±0.02</td>
<td>0.53±0.03</td>
<td>0.54±0.03</td>
</tr>
<tr>
<td>(Fucose/Protein) x 100</td>
<td>0.13±0.01</td>
<td>0.31±0.04</td>
<td>0.29±0.03</td>
</tr>
</tbody>
</table>
Table 7. Statistical comparison of the data of Table 5

<table>
<thead>
<tr>
<th>Ratio</th>
<th>Bovine/Porcine</th>
<th>Bovine/Avian</th>
<th>Porcine/Avian</th>
</tr>
</thead>
<tbody>
<tr>
<td>(H/P) x 100</td>
<td>7.4 8 0.001</td>
<td>5.5 9 0.001</td>
<td>8.9 9 0.001</td>
</tr>
<tr>
<td>(Hex/P) x 100</td>
<td>14.7 8 0.001</td>
<td>4.7 9 0.001</td>
<td>10.0 9 0.001</td>
</tr>
<tr>
<td>(SA/P) x 100</td>
<td>0.53 8 0.50</td>
<td>1.1 9 0.3</td>
<td>0.5 9 0.5</td>
</tr>
<tr>
<td>(F/P) x 100</td>
<td>9.5 8 0.001</td>
<td>13.1 9 0.001</td>
<td>1.1 9 0.03</td>
</tr>
</tbody>
</table>

(H/P) x 100 represents the ratio of hexosamine to protein concentration, times 100.

(Hex/P) x 100 represents the ratio of hexosamine concentration to protein concentration, times 100.

(SA/P) x 100 represents the ratio of sialic acid concentration to protein concentration, times 100.

(F/P) x 100 represents the ratio of fucose concentration to protein concentration, times 100.

df represents the number of degrees of freedom for the comparison being tested.

P represents the probability level at which the comparison is valid.
interesting facts may be seen. It appears that there is no significant difference between the sialic acid contents of the proteins of the three sera. The level of fucose in the porcine sera is significantly different from the level in the bovine sera, being almost twice as great. There is no significant difference between the level of fucose in the porcine and avian sera. The levels of hexosamines in serum proteins are significantly different. The avian serum proteins contain the most hexosamine, followed by the porcine, then the bovine serum protein. In the case of hexoses, the porcine serum has a ratio of hexose to protein nearly twice as much as do the bovine and avian sera. This difference is probably due in part to the higher content of fucose in the porcine serum.

C. ELECTROPHORESIS

Samples of bovine, porcine, and avian sera from mature females were subjected to electrophoresis in paper, cellulose acetate, agar and polyacrylamide gel supporting media. Results of these analyses are presented in Tables 8 to 13 and Figures 10 to 27.

1. Paper Electrophoresis

Samples of blood sera were examined by paper
electrophoresis (Sect. II,D,1). Following electrophoresis, electropherograms were stained for protein. Stained electropherograms were evaluated by scanning in a Spinco Model R Analytrol. A B-2 cam and two 500 m\textmu interference filters were used as suggested by Gaunce and Anastassiadis (71). Stained electropherograms were also cut into zones and the zones analyzed for hexosamine (Sect. II,D,1,c).

Values obtained for the distribution of protein and hexosamine are reported in Tables 8 and 9. Examples of typical densitometric scannings are presented in Figures 10 to 12.

The patterns of distribution of protein in paper electropherograms exhibit marked species differences. The bovine and porcine electropherograms each have five bands, corresponding to albumin, \(\alpha_1\)-globulin, \(\alpha_2\)-globulin, \(\beta\)-globulin and \(\gamma\)-globulin. The bovine sera contains a larger percentage of protein in the \(\alpha_1\)- and \(\alpha_2\)-globulins than does porcine sera. Conversely, porcine sera contains a larger percentage of protein in the \(\beta\)- and \(\gamma\)-globulins than does bovine sera.

Avian sera presents an entirely different pattern, the electropherograms exhibiting six zones, corresponding to albumin, \(\alpha_1\)-globulin, \(\alpha_2\)-globulin, \(\alpha_3\)-globulin,
\( \beta \)-globulin and \( \gamma \)-globulin. The avian sera contains a larger percentage of protein in the albumin zone than do the bovine or porcine sera. The avian \( \alpha_2 \)-globulin and \( \beta \)-globulin fractions contain a smaller per cent of the protein than do the corresponding fractions of bovine and porcine sera.

The distribution of hexosamine among the zones separated by paper electrophoresis also shows species variation. Porcine albumin contains less hexosamine than do bovine or avian albumins which have nearly equal quantities. The bovine \( \alpha_1 \)-globulin and \( \beta \)-globulin contain larger amounts of hexosamine than do the corresponding porcine globulins. These, in turn, contain more hexosamine than do the corresponding avian globulins.

Porcine \( \alpha_2 \)-globulin contains more hexosamine than does bovine \( \alpha_2 \)-globulin which contains more than the avian \( \alpha_2 \)-globulin.

Avian \( \gamma \)-globulin contains slightly more hexosamine than does porcine \( \gamma \)-globulin and both contain appreciably more hexosamine than the bovine \( \gamma \)-globulin.

Perhaps a better picture of species variation in electrophoretic distribution of hexosamine may be gained by examining the ratio of hexosamine to protein for the various zones. Since the distribution of protein is reported as
per cent of total it was decided to convert the data for hexosamine to per cent total as well and then to calculate the ratio. These calculated data are presented in Table 12. In most cases the ratios follow the same species variation as do values for hexosamine. In the case of $\beta$-globulins, however, the ratio of hexosamine to protein shows that the avian protein contains more hexosamine than the bovine protein. Also, bovine $\alpha_2$-globulin contains less hexosamine than does the avian globulin, when expressed as the ratio to protein. The sera of all three species appear to contain approximately the same amount of hexosamine per protein in the $\gamma$-globulin region, though the avian protein does contain somewhat less.

2. **Cellulose Acetate Electrophoresis**

Sera of the three species were examined by electrophoresis on cellulose acetate membrane strips. Electropherograms were stained for both protein and carbohydrate (Sect. II,D,2). It was found that sera easily gave reproducible electropherograms when stained for protein with amidoblack. Far greater difficulty was encountered in obtaining satisfactory carbohydrate stains.

Three different modifications of Schiff stains were
employed. The Sartorious method (234) was found to be more satisfactory than the Helena Laboratories method (286) or the method of Kelsey, de Graffenried and Donaldson (287).

Several different preparations of Schiff reagent were tested. Commercial Schiff reagents (Fisher Scientific Company, Fair Lawn, New Jersey, U.S.A.; Schiff reagent modified SO-S-32; Harleco, Philadelphia, Pa., Schiff reagent No. 2818 and Schiff reagent No. 6073) were tested and found to be less satisfactory than those prepared in this laboratory.

Several different basic fuchsins were employed in the preparation of the Schiff reagents (Fuchsin, basic, Coleman & Bell Co., Norwood, Ohio, U.S.A.; Basic Fuchsin 760154, F-98, Fuchsin basic red A-803, Fisher Scientific Company, Fair Lawn, New Jersey; Basic Fuchsin C-1762, Eastman Organic Chemicals, Rochester 3, New York; and Basic Fuchsin, special for Flagella stain, No. 719, Allied Chemical). Of the fuchsins tested the one produced by Allied Chemicals was found to give best results.

Different methods of preparation of the Schiff reagent were employed (234, 278, 286, 287). Of these, the method used (Sect. II,D,2,c) was found to be best.

Following electrophoresis and staining of strips,
densitometric evaluation was carried out. Initially both the Spinco Analytrol Model R with B-2 cam and two 500 m\(\mu\) interference filters and the Photovolt Densicord with 545 m\(\mu\) filters were employed for the scanning of the electropherograms. It was found that the Photovolt, a more versatile machine by virtue of its greater adjustability, was more suited to the evaluation of cellulose acetate electropherograms than was the Analytrol. The Photovolt scanner was used in all subsequent analyses.

To determine optimal settings for the Photovolt, a cellulose acetate electropherogram was scanned repeatedly at different scanner settings. The range setting of the scanner was always in the lowest possible position. The response setting was then varied between successive scans, adjusting the dark point and zero settings each time. Results of these trials are presented in Table 14.

It was found that for protein stained electropherograms the best response setting was position 7 or 8. In the case of glycoprotein staining it was found that response setting 1 to 3 had to be used. Lower or higher settings resulted in poorer scans due to the light coloration of the bands.

The distributions of protein and glycoprotein
(carbohydrate) following cellulose acetate electrophoresis are reported in Tables 8 and 9. The ratio of glycoprotein to protein was also determined (Table 12).

Examination of Table 8 reveals that there is a considerable difference in the electrophoretic distribution of protein following paper or cellulose acetate electrophoresis. In all cases the cellulose acetate electropherograms contain a greater percentage of protein in the albumin region than do the corresponding paper electropherograms. The $\alpha_2$-globulins and $\gamma$-globulins of all species exhibit a lower percentage of protein in the cellulose acetate electropherogram than in the paper electropherogram. The differences in the other zones are variable with species. For example, cellulose acetate electropherograms of bovine and avian sera show less protein in the $\alpha_1$-globulins than do the corresponding paper electropherograms. On the other hand, porcine serum electropherograms show more protein in cellulose acetate than paper.

These differences between electrophoretic distribution of protein in paper and cellulose acetate may largely be attributed to the use of different densitometers for the evaluation of the stained electropherograms. There may also be differences in the dye binding capacity of the proteins.
when on paper or on cellulose acetate. Another possible source of difference is the fact that the cellulose acetate electropherograms were scanned following clearing, that is, after the background had been made transparent, whereas the paper electropherograms were opaque. This would result in a greater variation between background and stained protein, on the cellulose acetate strips than on the paper. Thus, possibly, a greater resolution of the fainter bands may be obtained by use of the cellulose acetate strips.

The species variation in distribution of protein following electrophoresis in cellulose acetate strips is similar to that obtained by paper electrophoresis in most cases. Variations are shown in the $\beta$-globulins. The percentage distribution of protein in descending order of magnitude is bovine, porcine, avian for $\beta$-globulin on cellulose acetate but is porcine, bovine, avian for the same protein on paper. Possible reasons for these variations have been discussed.

Glycoprotein staining of cellulose acetate electropherograms reveals species differences in the electrophoretic distribution of protein bound carbohydrates. Porcine albumin and $\alpha_2$-globulin appears to contain more carbohydrate than do the corresponding bovine or avian proteins. Bovine
\(\alpha_1\)-globulin contains more carbohydrate than do either porcine or avian \(\alpha_1\)-globulin. Avian \(\beta\)-globulin and \(\gamma\)-globulin appear to contain more carbohydrate than do the bovine or porcine globulins.

The differences in hexosamine content of porcine and avian \(\alpha_1\)-globulin and of avian and bovine \(\alpha_2\)-globulin and \(\gamma\)-globulins are reversed when the carbohydrate to protein ratios (Table 12) are considered. The differences observed in the hexosamine contents of these zones had resulted from differences in protein content.

Comparisons of the distribution of hexosamine following paper electrophoresis and of the distribution of carbohydrate following cellulose acetate electrophoresis may be made. As both constituents are protein bound, it is useful to examine the relationship between the ratios hexosamine to protein and carbohydrate to protein (Table 12). Examination of this data reveals that the two distributions are not identical. In all cases, the albumin fraction of a given sera, separated by paper electrophoresis contains a higher percentage of hexosamine than the similar fraction, separated by cellulose acetate electrophoresis, contains carbohydrate. This difference was less in the case of porcine sera than in the other two. All \(\alpha_1\)-globulins had a higher
ratio of carbohydrate to protein than hexosamine to protein. Values for $\alpha_1$-globulin and $\alpha_2$-globulin are close for both methods. Variation is shown between the hexosamine and the carbohydrate distributions of $\beta$- and $\gamma$-globulins as determined by the two methods.

The ratios of these two ratios, hexosamine to protein and glycoprotein to protein, are presented in Table 13. This table shows the differences between the two distributions.

During the course of these experiments it was noted that the bovine serum albumin had a greater mobility than the porcine serum albumin which was greater than that of the avian albumin. This difference is evident in the photograph of a typical electrophoretic run (Figure 13) and in the densitometric tracings (Figures 14 to 19).

The conditions of electrophoresis were not held constant with respect to duration of the run, hence, some electrophoretic patterns exhibited greater movement of protein or of glycoprotein. To permit a comparison of different electrophoretic runs, tracings were adjusted for relative mobility with respect to albumin which was set at 100. Typical adjusted tracings are shown in Figures 17 to 19.
3. Polyacrylamide Gel Electrophoresis

As with paper and cellulose acetate electrophoresis, serum samples were examined for distribution following electrophoresis in polyacrylamide gels (Sect. II,D,4). The acrylamide gel electropherograms were stained for protein with amidoblack (Sect. II,D,4,d) and for glycoprotein by a periodic acid-Schiff stain (Sect. II,D,4,c). Originally the method of Keyser et al. (278) was employed for carbohydrate stain. The alcoholic solvents used in this procedure were found to produce an undesirable, variable, shrinkage in the gel. It was desirable to eliminate this shrinkage so that gels stained for carbohydrate could be compared with each other and with gels stained for protein. The method of Movshovich (270) initially used to stain agar gels was employed. The use of this method eliminated the alcoholic solvent and resulted in a marked decrease in the shrinkage of the gels.

Typical polyacrylamide gel electropherograms of bovine, porcine and avian sera are shown in Figures 20 and 21.

Evaluation of the acrylamide gel distributions was accomplished with the photovolt scanner as for cellulose acetate (Sect. III,C,2). Typical densitometer tracings
are shown in Figures 22 to 27. Tracings adjusted so as to remove effects of shrinkage are shown in Figures 25 to 27.

As for cellulose acetate electropherograms, the percentage distribution of protein (Table 8) and carbohydrate (Table 9) were calculated. The ratio of glycoprotein to protein was also calculated (Table 12).

The patterns of distribution presented following acrylamide gel electrophoresis are generally comparable to those obtained by the other methods. Differences are however, evident. These differences may largely be attributed to differences in separation due to the molecular sieve effect of the polyacrylamide gel.

4. Agar Electrophoresis

Electrophoresis in agar gels was employed to study the distribution of protein, hexosamine and sialic acid. Following electrophoresis, the gel was cut into segments and the proteins eluted (Sect. II,D,3). Eluates were analyzed for protein, hexosamine and sialic acid (Sect. II,C, 1,3,5b). Results of these experiments are presented in Tables 10 and 11.

Protein distribution obtained by agar electrophoresis was somewhat similar to that obtained by paper and cellulose
acetate electrophoresis. A larger percentage of protein was recovered in the albumin zone following agar electrophoresis than was found following paper, cellulose acetate or acrylamide electrophoresis. Smaller amounts of protein were found in the other zones following agar electrophoresis.

The same species variation shown for hexosamine distribution following paper electrophoresis (Sect. III,C,1) was shown in agar electrophoresis.

Species variations were evident in the distribution of sialic acid. Values of sialic acid to protein (Table 12) may be compared. All three species seem to have identical sialic acid contents in their albumin proteins. The bovine $\alpha_1$-globulin contains more sialic acid than the porcine or avian $\alpha_1$-globulins. On the other hand, the porcine $\alpha_2$-globulin appears to contain the most sialic acid, followed by the avian, then the bovine $\alpha_2$-globulins. In the case of the $\beta$- and $\gamma$-globulins it is the avian proteins which contain the most sialic acid, followed by the bovine, then the porcine proteins.

In general the sera of the two mammalian species contain their largest amounts of carbohydrate in the $\alpha_1$- and $\alpha_2$-globulins, whereas the avian sera has the carbohydrate
more evenly distributed, with largest quantities in the 
\( \alpha_3 \), \( \beta \) - and \( \gamma \)-globulins.

D. FRACTIONATION OF PLASMA

Grant et al. (77) working in this department iso-
lated purified, homogeneous M-1 and M-2 glycoproteins from porcine P-3 and P-4 fractions. At the same time they noted that while the P-1 and P-2 fractions contained smaller percentages of hexose and hexosamine, the total amount of these constituents in these fractions was very large. It was therefore decided to pursue this observation further and to study the distribution of hexose, hexosamine, sialic acid and fucose among the four fractional precipitates P-1, P-2, P-3 and P-4, as well as among the fractions obtained by the carboxymethyl-cellulose column chromatography of them.

Porcine blood was collected when animals were sacri-
ficed by cutting the jugular vein. Plasma was prepared from this blood. The blood collected was from sexually mature female Landrace pigs.

The porcine plasma was fractionated by sequential precipitation with ammonium sulphate (Sect. II,E.1). The precipitates obtained were analyzed for hexose, hexosamine,
Table 8. Distribution of protein (as per cent of total) in the electrophoretic zones of bovine, porcine and avian sera as obtained by paper (P), cellulose acetate (CA), agar (Ag) and acrylamide (A) electrophoreses

<table>
<thead>
<tr>
<th>Species Method</th>
<th>Pre-albumin</th>
<th>albumin</th>
<th>$\alpha_1$-globulin</th>
<th>$\alpha_2$-globulin</th>
<th>$\alpha_3$-globulin</th>
<th>$\beta$-globulin</th>
<th>$\gamma$-globulin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bovine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$p_1$</td>
<td>-</td>
<td>39.8±3.2</td>
<td>15.9±1.6</td>
<td>17.5±1.2</td>
<td>-</td>
<td>16.9±1.1</td>
<td>9.8±0.8</td>
</tr>
<tr>
<td>CA$^1$</td>
<td>-</td>
<td>43.3±4.0</td>
<td>11.9±1.2</td>
<td>17.4±0.5</td>
<td>-</td>
<td>20.9±2.1</td>
<td>6.5±0.6</td>
</tr>
<tr>
<td>Ag$^1$</td>
<td>-</td>
<td>51.3±5.9</td>
<td>10.8±2.2</td>
<td>14.3±1.9</td>
<td>-</td>
<td>15.6±2.0</td>
<td>8.0±1.2</td>
</tr>
<tr>
<td>A$^1$</td>
<td>0.6±0.1</td>
<td>49.1±4.5</td>
<td>12.7±1.3</td>
<td>15.6±2.2</td>
<td>-</td>
<td>19.2±2.3</td>
<td>2.8±0.2</td>
</tr>
<tr>
<td><strong>Porcine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$p_1$</td>
<td>-</td>
<td>30.8±2.9</td>
<td>16.8±1.7</td>
<td>7.8±0.6</td>
<td>-</td>
<td>20.0±1.8</td>
<td>24.6±2.2</td>
</tr>
<tr>
<td>CA$^1$</td>
<td>-</td>
<td>37.9±3.7</td>
<td>19.4±1.0</td>
<td>6.9±0.6</td>
<td>-</td>
<td>15.6±1.2</td>
<td>20.2±2.1</td>
</tr>
<tr>
<td>Ag$^1$</td>
<td>-</td>
<td>43.2±4.7</td>
<td>8.7±0.9</td>
<td>6.9±0.8</td>
<td>-</td>
<td>17.2±2.3</td>
<td>24.0+</td>
</tr>
<tr>
<td>A$^1$</td>
<td>0.5±0.1</td>
<td>41.2±4.2</td>
<td>11.7±1.0</td>
<td>7.5±0.5</td>
<td>-</td>
<td>18.9±2.1</td>
<td>20.2±2.0</td>
</tr>
<tr>
<td><strong>Avian</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$p_1$</td>
<td>-</td>
<td>37.6±3.1</td>
<td>15.2±0.4</td>
<td>7.1±0.8</td>
<td>8.4±0.9</td>
<td>10.6±1.0</td>
<td>21.1±3.0</td>
</tr>
<tr>
<td>CA$^1$</td>
<td>-</td>
<td>46.6±3.7</td>
<td>11.4±1.1</td>
<td>6.8±0.8</td>
<td>5.0±0.4</td>
<td>13.2±1.1</td>
<td>17.0±2.0</td>
</tr>
<tr>
<td>Ag$^2$</td>
<td>-</td>
<td>48.3±5.6</td>
<td>10.9±2.1</td>
<td>6.6±0.7</td>
<td>7.3±0.8</td>
<td>12.6±2.0</td>
<td>14.3±1.9</td>
</tr>
<tr>
<td>A$^2$</td>
<td>0.5±0.1</td>
<td>47.5±4.2</td>
<td>11.4±1.5</td>
<td>6.2±0.9</td>
<td>6.1±0.4</td>
<td>10.2±1.2</td>
<td>18.1±2.1</td>
</tr>
</tbody>
</table>

1Values represent mean value ± standard deviation of five different animals.

2Values represent mean value ± standard deviation of six different animals.
Table 9. Distribution of glycoprotein (as per cent of total) in the electrophoretic zones of bovine, porcine and avian sera as obtained by cellulose acetate (CA) and acrylamide (A) electrophoreses

<table>
<thead>
<tr>
<th>Electrophoretic Zone</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bovine</td>
<td></td>
<td>Porcine</td>
<td></td>
<td>Avian</td>
</tr>
<tr>
<td></td>
<td>C.A.</td>
<td>A</td>
<td>C.A.</td>
<td>A</td>
<td>C.A.</td>
</tr>
<tr>
<td>Pre-albumin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Albumin</td>
<td>2.5±0.4</td>
<td>4.3</td>
<td>11.2±1.4</td>
<td>7.5</td>
<td>4.8±0.9</td>
</tr>
<tr>
<td>α₁-globulin</td>
<td>55.6±8.2</td>
<td>38.2</td>
<td>43.7±4.9</td>
<td>31.2</td>
<td>32.1±3.7</td>
</tr>
<tr>
<td>α₂-globulin</td>
<td>16.0±2.1</td>
<td>19.7</td>
<td>20.0±2.6</td>
<td>18.6</td>
<td>13.1±1.6</td>
</tr>
<tr>
<td>α₃-globulin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>8.3±1.1</td>
</tr>
<tr>
<td>β-globulin</td>
<td>9.9±1.2</td>
<td>14.4</td>
<td>18.1±2.5</td>
<td>22.5</td>
<td>23.8±2.8</td>
</tr>
<tr>
<td>γ-globulin</td>
<td>16.0±2.5</td>
<td>23.1</td>
<td>7.0±1.1</td>
<td>19.8</td>
<td>17.9±2.4</td>
</tr>
</tbody>
</table>

¹Values represent mean value ± standard deviation for five animals.
²Values represent mean value for two animals.
³Values represent mean value for three animals.
⁴Values represent mean value + standard deviation for six animals.
Table 10. Distribution of hexosamine (mean concentration ± standard deviation) in electrophoretic zones of bovine, porcine and avian series agar (AG) electrophoreses

<table>
<thead>
<tr>
<th>Protein</th>
<th>BOVINE P (mg/100 ml)</th>
<th>BOVINE AG (mg/100 ml)</th>
<th>PORCINE P (mg/100 ml)</th>
<th>PORCINE AG (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>18.7±1.4</td>
<td>21.4</td>
<td>10.0±0.5</td>
<td>14.0</td>
</tr>
<tr>
<td>α₁-globulin</td>
<td>26.8±2.4</td>
<td>30.7</td>
<td>29.4±2.2</td>
<td>41.3</td>
</tr>
<tr>
<td>α₂-globulin</td>
<td>12.6±1.4</td>
<td>14.4</td>
<td>11.4±1.3</td>
<td>16.0</td>
</tr>
<tr>
<td>α₃-globulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-globulin</td>
<td>19.2±1.9</td>
<td>22.0</td>
<td>12.6±1.2</td>
<td>17.7</td>
</tr>
<tr>
<td>γ-globulin</td>
<td>10.0±1.2</td>
<td>11.5</td>
<td>7.8±0.5</td>
<td>11.0</td>
</tr>
<tr>
<td>Total</td>
<td>87.3±9.5</td>
<td>100</td>
<td>71.2±5.7</td>
<td>100</td>
</tr>
</tbody>
</table>


ion of hexosamine (mean concentration ± standard deviation and %) in the
serotic zones of bovine, porcine and avian sera as obtained by paper (P) and
1 electrophoreses

<table>
<thead>
<tr>
<th></th>
<th>BOVINE</th>
<th></th>
<th>PORCINE</th>
<th></th>
<th>AVIAN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P</td>
<td>AG</td>
<td>P</td>
<td>AG</td>
<td>P</td>
</tr>
<tr>
<td>mg/100</td>
<td>ml</td>
<td>%</td>
<td>mg/100</td>
<td>ml</td>
<td>%</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td></td>
<td>%</td>
<td></td>
<td>%</td>
</tr>
<tr>
<td>1.4</td>
<td>21.4</td>
<td>10.0±0.5</td>
<td>14.0</td>
<td>7.9±0.6</td>
<td>9.0</td>
</tr>
<tr>
<td>2.4</td>
<td>30.7</td>
<td>29.4±2.2</td>
<td>41.3</td>
<td>17.1±1.9</td>
<td>19.4</td>
</tr>
<tr>
<td>1.4</td>
<td>14.4</td>
<td>11.4±1.3</td>
<td>16.0</td>
<td>25.2±2.9</td>
<td>28.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.9</td>
<td>22.0</td>
<td>12.6±1.2</td>
<td>17.7</td>
<td>16.3±1.3</td>
<td>18.5</td>
</tr>
<tr>
<td>1.2</td>
<td>11.5</td>
<td>7.8±0.5</td>
<td>11.0</td>
<td>21.6±1.9</td>
<td>24.5</td>
</tr>
<tr>
<td>3.5</td>
<td>100</td>
<td>71.2±6.7</td>
<td>100</td>
<td>88.1±10.2</td>
<td>100</td>
</tr>
</tbody>
</table>
Table 11. Distribution of hexosamine (H) and sialic acid (SA) (mg per 100 ml of sera) and ratio of hexosamine to sialic acid (H/SA) in the electrophoretic zones of bovine, porcine and avian sera as obtained by agar electrophoreses

<table>
<thead>
<tr>
<th>Electrophoretic Zone</th>
<th>Bovine</th>
<th>Porcine</th>
<th>Avian</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H</td>
<td>SA</td>
<td>H/SA</td>
</tr>
<tr>
<td>Albumin</td>
<td>10.0±0.5</td>
<td>3.4±0.2</td>
<td>2.94</td>
</tr>
<tr>
<td>α₁-globulin</td>
<td>29.4±2.2</td>
<td>15.5±1.3</td>
<td>1.90</td>
</tr>
<tr>
<td>α₂-globulin</td>
<td>11.4±1.3</td>
<td>6.7±0.4</td>
<td>1.70</td>
</tr>
<tr>
<td>α₃-globulin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-globulin</td>
<td>12.6±1.2</td>
<td>4.4±0.5</td>
<td>2.86</td>
</tr>
<tr>
<td>γ-globulin</td>
<td>7.8±0.5</td>
<td>2.1±0.1</td>
<td>3.71</td>
</tr>
</tbody>
</table>
Table 12. Ratios of hexosamine to protein (H/P), glycoprotein (SA/P) in the electrophoretic zones of sera as obtained by paper (P), cellulose acetate electrophoreses

| Electro- | BOVINE | PORC |         |         |         |         |         |
| phoretic |        |      |         |         |         |         |         |
| zone     | H/P    | G/P  | SA/P    | H/P    | G/P    | SA/P    |
|          | P      | AG   | CA      | A      | AG     | CA      |
| Pre-albumin |        |      |         |         |         |         |         |
| Albumin  | 0.58   | 0.27 | 0.06    | 0.09   | 0.07   | 0.29    | 0.19    | 0.30 |
| $\alpha_1$-globulin | 2.16   | 3.82 | 4.67    | 3.01   | 1.44   | 1.15    | 2.98    | 2.25 |
| $\alpha_2$-globulin | 0.87   | 1.12 | 0.92    | 1.26   | 0.47   | 3.67    | 4.17    | 2.90 |
| $\alpha_3$-globulin |        |      |         |         |         |         |         |      |
| $\beta$-globulin  | 1.05   | 1.13 | 0.47    | 0.75   | 0.28   | 0.93    | 1.15    | 1.16 |
| $\gamma$-globulin | 1.34   | 1.38 | 2.46    | 8.25   | 0.26   | 1.00    | 0.71    | 0.35 |
to protein (H/P), glycoprotein to protein (G/P) and sialic acid to
\[ \text{protein (H/P), glycoprotein to protein (G/P) and sialic acid to} \]
\[ \text{electrophoretic zones of bovine (B), porcine (P) and avian (A)} \]
\[ \text{paper (P), cellulose acetate (CA), agar (AG) and acrylamide (A)} \]

<table>
<thead>
<tr>
<th></th>
<th>SA/P</th>
<th>H/P</th>
<th>G/P</th>
<th>SA/P</th>
<th>H/P</th>
<th>G/P</th>
<th>SA/P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>AG</td>
<td>P</td>
<td>AG</td>
<td>CA</td>
<td>A</td>
<td>AG</td>
</tr>
<tr>
<td></td>
<td>0.09</td>
<td>0.07</td>
<td>0.29</td>
<td>0.19</td>
<td>0.30</td>
<td>0.18</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>3.01</td>
<td>1.44</td>
<td>1.15</td>
<td>2.98</td>
<td>2.25</td>
<td>2.67</td>
<td>1.37</td>
</tr>
<tr>
<td></td>
<td>1.26</td>
<td>0.47</td>
<td>3.67</td>
<td>4.17</td>
<td>2.90</td>
<td>2.48</td>
<td>1.61</td>
</tr>
<tr>
<td></td>
<td>1.88</td>
<td>2.11</td>
<td>0.60</td>
<td>6.10</td>
<td>0.55</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>0.28</td>
<td>0.93</td>
<td>1.15</td>
<td>1.16</td>
<td>1.19</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>8.25</td>
<td>0.26</td>
<td>1.00</td>
<td>0.71</td>
<td>0.35</td>
<td>1.02</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>0.73</td>
<td>0.72</td>
<td>1.05</td>
<td>0.77</td>
<td>0.31</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 13. Ratio of hexosamine (%) following paper electrophoresis to glycoprotein (%) following cellulose acetate electrophoresis for the electrophoretic zones of bovine, porcine and avian sera following paper and cellulose acetate electrophoresis

<table>
<thead>
<tr>
<th>Protein</th>
<th>Bovine</th>
<th>Porcine</th>
<th>Avian</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>9.00</td>
<td>0.97</td>
<td>6.00</td>
</tr>
<tr>
<td>$\alpha_1$-globulin</td>
<td>0.41</td>
<td>0.51</td>
<td>0.35</td>
</tr>
<tr>
<td>$\alpha_2$-globulin</td>
<td>0.89</td>
<td>1.27</td>
<td>1.24</td>
</tr>
<tr>
<td>$\alpha_3$-globulin</td>
<td>-</td>
<td>-</td>
<td>3.13</td>
</tr>
<tr>
<td>$\beta$-globulin</td>
<td>2.77</td>
<td>0.80</td>
<td>1.30</td>
</tr>
<tr>
<td>$\gamma$-globulin</td>
<td>0.48</td>
<td>2.86</td>
<td>0.70</td>
</tr>
</tbody>
</table>

Table 14. Variations in protein distribution in cellulose acetate electrophorogram of porcine sera with changes in response settings

<table>
<thead>
<tr>
<th>Electrophoretic Zone</th>
<th>Response Settings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Albumin</td>
<td>28.2</td>
</tr>
<tr>
<td>$\alpha_1$-globulin</td>
<td>20.6</td>
</tr>
<tr>
<td>$\alpha_2$-globulin</td>
<td>11.8</td>
</tr>
<tr>
<td>$\beta$-globulin</td>
<td>17.5</td>
</tr>
<tr>
<td>$\gamma$-globulin</td>
<td>21.7</td>
</tr>
</tbody>
</table>
Figure 10. Electrophoretic distribution of bovine serum proteins following paper electrophoresis.
Figure 11. Electrophoretic distribution of porcine serum proteins following paper electrophoresis.
Figure 12. Electrophoretic distribution of avian serum proteins following paper electrophoresis.
Figure 13. Cellulose acetate electropherograms of bovine (1), porcine (3) and avian (5) sera stained for protein and of bovine (2), porcine (4) and avian (6) sera stained for glucoprotein.
Figure 14. Electrophoretic distribution of bovine serum proteins following cellulose acetate electrophoresis.
Figure 15. Electrophoretic distribution of porcine serum proteins following cellulose acetate electrophoresis.
Figure 16. Electrophoretic distribution of avian serum proteins following cellulose acetate electrophoresis.
Figure 17. Electrophoretic distribution of bovine serum protein and glycoprotein following cellulose acetate electrophoresis.
Figure 18. Electrophoretic distribution of porcine serum protein and glycoprotein following cellulose acetate electrophoresis.
Figure 19. Electrophoretic distribution of avian serum protein and glycoprotein following cellulose acetate electrophoresis.
Figure 20. Acrylamide gel electropherogram of bovine (1), porcine (2) and avian (3) sera stained for protein.
Figure 21. Acrylamide gel electropherogram of bovine (1), porcine (2) and avian (3) sera stained for glycoprotein.
Figure 21. Acrylamide gel electropherogram of bovine (1), porcine (2) and avian (3) sera stained for glycoprotein.
Figure 22. Electrophoretic distribution of bovine serum proteins following polyacrylamide gel electrophoresis.
Figure 23. Electrophoretic distribution of porcine serum proteins following polyacrylamide gel electrophoresis.
Figure 24. Electrophoretic distribution of avian serum proteins following polyacrylamide gel electrophoresis.
Figure 25. Electrophoretic distribution of bovine serum protein and glycoprotein following polyacrylamide gel electrophoresis.
Figure 26. Electrophoretic distribution of porcine serum protein and glycoprotein following polyacrylamide gel electrophoresis.
Figure 27. Electrophoretic distribution of avian serum protein and glycoprotein following polyacrylamide gel electrophoresis.
sialic acid and fucose. Yields and compositions of the precipitates are reported in Table 15.

The largest yield of precipitate was in the P-1 fraction, the smallest in the P-4 fraction. Altogether 4.505 g of protein per liter of plasma were recovered. This represents a recovery of 55.6% of the plasma proteins. It was suspected that a large part of the protein was lost during dialysis. Qualitative tests of the water against which the precipitates were dialyzed showed the presence of proteins. Little protein remained in the supernatant following precipitation of P-4.

The recovery of hexose was the lowest of the components studied, being only 22.9%. This is as would be expected, as a large amount of hexose is present in the plasma in the free form, i.e. not bound to protein.

Recoveries of hexosamine (62.4%) and fucose (66.3%) were both high in comparison to the other constituents. It would appear that the protein lost during dialysis contained less carbohydrate than the proteins recovered.

Recoveries of sialic acid were somewhat lower than those for hexosamine and fucose. This likely reflects the fact that sialic acid, which is terminal in the glycoprotein molecule, is easily split off.
The P-1, P-2, P-3, and P-4 fractions and porcine sera were studied by cellulose acetate and acrylamide electrophoresis. Photographs of electropherograms are shown (Figures 28 - 30). In most cases the electropherograms, especially for glycoprotein, did not stain sufficiently well to permit densitometric evaluation. The electropherograms of the P-1 fraction showed that it contained all bands present of the serum. Their relative distributions seemed to exhibit some differences.

It appears that the P-2 fraction contains three main components, corresponding to albumin, $\alpha_2$- and $\beta$-globulin. The carbohydrate stained cellulose acetate electropherogram revealed one faint band in the $\alpha_1$-globulin region. This band was not visible in the photographs. The acrylamide gel electropherogram reveals a carbohydrate component corresponding to the third major zone of the electropherogram, $\beta$-globulin.

The P-3 fraction, stained for protein, reveals three major fractions in the cellulose acetate electropherogram and four in the acrylamide electropherogram. The electropherograms stained for glycoprotein reveal only one major band, corresponding to the third protein band in acrylamide, and the third band in cellulose acetate.
The P-4 fraction showed only one band in the cellulose acetate electropherogram, corresponding to $\alpha_1$-globulin, and three bands in the acrylamide electropherogram, corresponding to albumin and two others. The glycoprotein stain of the cellulose acetate electropherogram reveals two bands corresponding to the $\alpha_1$-globulins. No distinct bands were evident in the acrylamide electropherogram.

From this data it would appear that all four fractional precipitates contain glycoproteins. The P-1 appears to contain all those found in the whole sera. This would suggest the possibility that they are brought down in this fraction by co-precipitation. The P-2 appears to contain glycoproteins migrating with the mobility of $\alpha_1$-globulins. The P-3 fraction contains glycoproteins with mobilities similar to the $\gamma$-globulins. The P-4 fraction contains at least two glycoproteins with the mobility of the $\alpha_1$-globulins. The P-1 thus appears to contain all glycoproteins of the sera while P-2, P-3 and P-4 each contain different glycoproteins.

Following this analysis, the fractions were chromatographed on columns of carboxymethyl-cellulose (Sect. II,E, 2). Originally a column of 2.8 x 35 cm and a sample of 2.0 g or less were employed. In chromatography of P-1 and P-2
especially, this resulted in recovery of some very small fractions. It was decided to increase the sample size to 5.0 g. To accommodate this increase in sample size the column was increased to 3.5 x 45 cm. Similar separations were obtained with both columns.

In all cases chromatography produced one peak per elution buffer (Figures 31-34). Recovery from the column was only 58 to 68%. An attempt to elute more proteins was made using a fourth elution buffer of pH 8.0. This resulted in the elution of a fourth peak containing little protein or carbohydrate. It thus appears that not much protein is remaining on the column. In all probability most of the losses occur during dialysis.

The fractions obtained following chromatography were analyzed for hexose, hexosamine, sialic acid and fucose (Tables 16-19). In all cases the recovery of these compounds was less than the recovery of protein. Lowest recoveries were of sialic acid. Recoveries were generally higher in the P-2 fraction and lower in the other fractions.

The fractions containing the highest percentage of constituents were the P-3-B and the P-4-A and B. The P-3-B and P-4-A are the source of the M-1 and M-2 glycoproteins isolated by Grant et al. (77).
If total concentration (per 100 ml of serum or per gram of fraction applied) is considered, different fractions are high in the constituents. For example, fractions P-1-B, P-2-B, P-3-A, P-3-B and P-4-C are all relatively high in hexose. These same fractions as well as P-4-A and P-4-B are high in hexosamine. Only fractions P-1-C and P-3-B and P-4-B are high in sialic acid, while only P-3-B and P-3-C and P-4-C are high in fucose.

If the high concentration of hexose and hexosamine in the P-1-B, P-2-B, P-3-A and P-4-B and P-4-C indicates the presence of glycoproteins, then these glycoproteins must have less sialic acid and fucose than the P-4-A and P-3-B (M-1 and M-2 glycoproteins). All fractions isolated were examined by cellulose acetate electrophoresis at pH 8.6. All fractions proved to be heterogeneous to some extent. The fractions of P-1 and P-2 as well as P-3-A, P-3-C and P-4-C all showed from three to five bands. P-3-B, P-4-A and P-4-B showed from two to three bands. Larger quantities of the fractions must be isolated and attempts must be made to isolate homogeneous proteins from them, so that their properties may be studied.

E. INCORPORATION OF RADIOACTIVE GLUCOSAMINE

Three mature male and one mature female White Leghorns
Table 15. Yield and composition of fractional precipitates of porcine plasma

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Yield (g/100 ml)</th>
<th>Hexosamine Total (mg/100 ml) %</th>
<th>Hexose Total (mg/100 ml) %</th>
<th>Sialic Acid Total (mg/100 ml) %</th>
<th>Fucose Total (mg/100 ml) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-1</td>
<td>3.4</td>
<td>1.11</td>
<td>37.7</td>
<td>1.15</td>
<td>0.43</td>
</tr>
<tr>
<td>P-2</td>
<td>1.0</td>
<td>1.29</td>
<td>12.9</td>
<td>1.72</td>
<td>0.83</td>
</tr>
<tr>
<td>P-3</td>
<td>0.1</td>
<td>3.99</td>
<td>4.0</td>
<td>5.83</td>
<td>1.91</td>
</tr>
<tr>
<td>P-4</td>
<td>0.005</td>
<td>5.31</td>
<td>0.3</td>
<td>6.65</td>
<td>0.5</td>
</tr>
<tr>
<td>Total</td>
<td>4.505</td>
<td>1.22</td>
<td>54.9</td>
<td>1.38</td>
<td>0.56</td>
</tr>
<tr>
<td>Serum levels</td>
<td>8.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Recovery</td>
<td>55.6</td>
<td>62.4</td>
<td>22.9</td>
<td>59.3</td>
<td>66.3</td>
</tr>
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</table>
Table 16. Yield and composition of fractions obtained by carboxymethyl cellulose chromatography of the P-1 precipitate of porcine plasma

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Yield (g/g)</th>
<th>Hexose %</th>
<th>Hexosamine %</th>
<th>Sialic Acid %</th>
<th>Fucose %</th>
<th>mg/g</th>
<th>mg/g</th>
<th>mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.0485</td>
<td>1.32</td>
<td>0.64</td>
<td>1.39</td>
<td>0.62</td>
<td>1.27</td>
<td>0.62</td>
<td>1.12</td>
</tr>
<tr>
<td>B</td>
<td>0.0015</td>
<td>2.25</td>
<td>0.03</td>
<td>3.41</td>
<td>0.05</td>
<td>1.03</td>
<td>0.02</td>
<td>1.02</td>
</tr>
<tr>
<td>C</td>
<td>0.5175</td>
<td>1.28</td>
<td>6.62</td>
<td>0.54</td>
<td>2.79</td>
<td>0.39</td>
<td>2.02</td>
<td>0.24</td>
</tr>
<tr>
<td>D</td>
<td>0.0192</td>
<td>1.29</td>
<td>0.25</td>
<td>0.24</td>
<td>0.05</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>58.67</td>
<td>7.54</td>
<td>3.56</td>
<td>2.66</td>
<td>1.80</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Recovery</td>
<td>58.7</td>
<td>65.6</td>
<td>32.1</td>
<td>62.0</td>
<td>61.8</td>
<td></td>
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</tbody>
</table>
Table 17. Yield and composition of fractions obtained by carboxymethyl cellulose chromatography of the P-2 precipitate of porcine plasma

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Yield (g/g)</th>
<th>Hexose</th>
<th>%</th>
<th>Hexosamine</th>
<th>%</th>
<th>Sialic Acid</th>
<th>%</th>
<th>Fucose</th>
<th>%</th>
<th>mg/g</th>
<th>mg/g</th>
<th>mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
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<td>0.18</td>
<td>1.23</td>
<td>0.19</td>
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<td>0.02</td>
<td>0.13</td>
<td>0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.0721</td>
<td>2.15</td>
<td>1.55</td>
<td>2.96</td>
<td>2.13</td>
<td>2.04</td>
<td>1.47</td>
<td>1.09</td>
<td>0.79</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.5930</td>
<td>0.95</td>
<td>5.63</td>
<td>0.83</td>
<td>7.92</td>
<td>0.32</td>
<td>1.90</td>
<td>0.24</td>
<td>1.42</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.6805</td>
<td>7.18</td>
<td>7.24</td>
<td>3.39</td>
<td>2.23</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Recovery</td>
<td>68.1</td>
<td>41.7</td>
<td>56.1</td>
<td>40.8</td>
<td>63.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 18. Yield and composition of fractions obtained by carboxymethyl cellulose chromatography of the P-3 precipitate of porcine plasma

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Yield (g/g)</th>
<th>Hexose</th>
<th>Hexosamine</th>
<th>Sialic Acid</th>
<th>Fucose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>%</td>
<td>mg/g</td>
<td>%</td>
<td>mg/g</td>
</tr>
<tr>
<td>A</td>
<td>0.0074</td>
<td>6.72</td>
<td>0.50</td>
<td>4.73</td>
<td>0.35</td>
</tr>
<tr>
<td>B</td>
<td>0.0215</td>
<td>5.59</td>
<td>1.20</td>
<td>4.56</td>
<td>0.98</td>
</tr>
<tr>
<td>C</td>
<td>0.5845</td>
<td>1.57</td>
<td>9.18</td>
<td>2.12</td>
<td>12.39</td>
</tr>
<tr>
<td>Total</td>
<td>0.6134</td>
<td>10.88</td>
<td>13.72</td>
<td>6.72</td>
<td>7.89</td>
</tr>
<tr>
<td>% Recovery</td>
<td>61.3</td>
<td>18.8</td>
<td>34.3</td>
<td>35.4</td>
<td>46.4</td>
</tr>
</tbody>
</table>
Table 19. Yield and composition of fractions obtained by carboxymethyl cellulose chromatography of the P-4 precipitate of porcine plasma

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Yield (g/g)</th>
<th>Hexose (%)</th>
<th>Hexosamine (%)</th>
<th>Sialic Acid (%)</th>
<th>Fucose (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mg/g</td>
<td>mg/g</td>
<td>mg/g</td>
<td>mg/g</td>
</tr>
<tr>
<td>A</td>
<td>0.0071</td>
<td>13.59</td>
<td>0.96</td>
<td>11.72</td>
<td>6.52</td>
</tr>
<tr>
<td>B</td>
<td>0.0119</td>
<td>2.61</td>
<td>0.91</td>
<td>6.39</td>
<td>0.76</td>
</tr>
<tr>
<td>C</td>
<td>0.6327</td>
<td>2.95</td>
<td>18.66</td>
<td>2.15</td>
<td>13.60</td>
</tr>
<tr>
<td>Total</td>
<td>0.6517</td>
<td>20.53</td>
<td>15.19</td>
<td>41.34</td>
<td>48.75</td>
</tr>
<tr>
<td>% Recovery</td>
<td>65.2</td>
<td>34.2</td>
<td>25.3</td>
<td>41.3</td>
<td>30.5</td>
</tr>
</tbody>
</table>
Figure 28. Cellulose acetate electropherogram of porcine sera (1), P-1 (2), P-2 (3), P-3 (4) and P-4 (5) plasma fractions stained for protein.
Figure 29. Cellulose acetate electropherogram of porcine sera (1), P-1 (2), P-2 (3), P-3 (4) and P-4 (5) plasma fractions stained for glycoprotein.
Figure 30. Acrylamide gel electropherogram of porcine plasma fractions P-1 (5), P-2 (6), P-3 (7) and P-4 (8) stained for protein, and P-1 (1), P-2 (2), P-3 (3) and P-4 (4) stained for glycoprotein.
Figure 31. Discontinuous elution chromatography of a porcine P-1 fraction on CM-cellulose
Figure 32. Discontinuous elution chromatography of a porcine P-2 fraction on CM-cellulose.
Figure 33. Discontinuous elution chromatography of a porcine P-3 fraction on CM-cellulose.
Figure 34. Discontinuous elution chromatography of a porcine P-4 fraction on CM-cellulose.
were injected with approximately 50 microcuries each of Glucosamine-1-\textsuperscript{14}C (New England Nuclear Corporation). Samples of blood were taken at various intervals following injection. Serum and in one case plasma was prepared from these blood samples. The amount of activity present in the free and protein bound forms was determined. These data were plotted against time to obtain incorporation curves (Figures 35 and 36).

It was found that incorporation curves varied widely from bird to bird. In general the protein bound activity reached a maximum from 2 to 10 hours after injection, following which it dropped off at varying rates.

In one experiment a rooster was injected intraperitoneally. A lag of a few hours was observed in the curves of incorporation of both free and protein bound activity as compared to the curves for birds injected intravenously. In one other experiment there was not a rapid rise in the increase of free activity, instead there was a lag in the rise similar to that observed with the intraperitoneal injection. This was taken as an indication that the attempted intravenous injection had been a failure and that substantial amounts of the activity had been deposited underneath the skin, adjacent to the vein.

Sera samples containing the greatest amounts of
protein bound activity were studied by agar electrophoresis (Sect. II,E,3). Following electrophoresis the gel was cut into segments about $\frac{1}{4}''$ wide and the proteins were eluted. Concentration of protein and hexosamine in the eluates was determined (Sect. II,C,1.5b). Activity of each eluate was also determined (Sect. II,F,1). These values were plotted against distance migrated to give a pattern of distribution of protein, hexosamine and activity (Figure 37). It is immediately evident that the curves of hexosamine and activity are not parallel. This would suggest that some glycoproteins have a greater turnover rate and thus contain more activity at the time the sample was taken. It is also possible that all activity is not present as hexosamine. This question may be resolved by running similar electrophoretic experiments, or autoradiographic experiments, on sera samples taken at different times following injection. Also serum may be obtained and the activity associated with hexose, hexosamine, sialic acid and fucose may be determined.
Figure 35. Incorporation of activity into rooster serum following intraperitoneal injection of $1^{-14}C$-glucosamine.
Figure 36. Incorporation of activity into hen serum following intravenous injection of \( l^{14}C \)-glucosamine.
Figure 37. Electrophoretic distribution of protein, hexosamine and activity in rooster serum following agar electrophoresis.
IV. SUMMARY

1. An experiment was conducted to verify the unpublished observations of Mr. A.C. Murray of this department, that the precision of the hexosamine determination was improved by increasing the size of the reaction vessel employed. This experimenter was unable to verify the observation.

2. The serum concentrations of protein, hexosamine, sialic acid and fucose of the bovine, porcine and avian species were examined. Samples were drawn at random throughout the year from non-pregnant or non-laying females. Any diurnal variation as well as interanimal variation is thus included in the overall variation of the experiment. Results were expressed both on an absolute concentration basis and on a per gram protein basis.

Porcine serum proteins contained significantly more hexose and fucose than did the bovine or avian proteins. The avian serum proteins contained significantly more hexosamine than did the bovine or porcine proteins.

3. Paper electrophoresis of the serum proteins separated the bovine and porcine proteins into five zones and the avian into six zones. Species differences were evident in
the patterns of distribution. The bovine sera contained a greater percentage of protein in the \( \alpha_1 \)- and \( \alpha_2 \)-globulin zones, while the porcine sera contained a greater percentage in the \( \beta \)- and \( \gamma \)-globulins, and the avian sera contained a greater percentage in the albumin, than did the corresponding zones of the other species.

Porcine albumin contained less hexosamine than did the bovine or avian. The bovine \( \alpha_1 \)-globulin and \( \beta \)-globulin contained larger amounts than did the porcine globulin, which in turn contained more than did the avian globulin. Porcine \( \alpha_2 \)-globulin contained more hexosamine than did the bovine or avian \( \alpha_2 \)-globulins. Of the three species, bovine \( \gamma \)-globulin contained less hexosamine than did the other two.

In most cases similar species variations were evident when results were expressed as the ratio of per cent hexosamine to per cent protein for a given zone. In the \( \beta \)-globulins however, the avian protein had a higher hexosamine to protein ratio than did the bovine or porcine. Bovine \( \alpha_2 \)-globulin, as well, had a lower ratio than did the avian globulin.

4. Cellulose acetate electrophoresis revealed different patterns than did paper electrophoresis. Cellulose acetate
electropherograms contained a greater proportion of protein in the albumin zones, and a smaller proportion in the \( \alpha_2 \)- and \( \gamma \)-globulin zones, than corresponding paper electropherograms. Differences were attributed to differences in dye binding capacity of the proteins when on paper and cellulose acetate, differences in staining techniques, especially due to clearing of the cellulose acetate strips, and to differences in densitometric evaluation.

Species variation in protein distributions are similar to those obtained by paper electrophoresis, with the exception of the \( \beta \)-globulins.

Species differences were evident in the distributions of carbohydrate, revealed by a periodic acid-Schiff stain. Porcine albumin and \( \alpha_2 \)-globulin contained more carbohydrate than did the corresponding bovine or avian zones. Bovine \( \alpha_1 \)-globulin contained more carbohydrate than did the corresponding bovine or porcine globulins.

Distributions of hexosamine following paper electrophoresis and of carbohydrates following cellulose acetate electrophoresis were not identical. In all cases the percentage of hexosamine in the albumin was greater than the percentage of carbohydrate in the same zone. The reverse was true for the \( \alpha_1 \)-globulins and the \( \alpha_2 \)-globulins.
5. Electrophoretic distribution of protein and carbohydrate following electrophoresis on polyacrylamide gels was studied. Distribution patterns were comparable to those obtained by other methods. Differences which were evident were attributed to the molecular sieve effect of the polyacrylamide gels.

6. Electrophoretic distribution of protein, hexosamine and sialic acids were studied following electrophoresis in agar gels. Protein distributions obtained in this manner were comparable to those obtained on cellulose acetate and paper, though a larger percentage of protein was found in the albumin zone of the agar electropherograms.

   The same species variations in hexosamine distribution following paper electrophoresis were found following agar electrophoresis.

   Species differences were evident in sialic acid distributions among the electrophoretic zones with the exception of the albumin. Bovine $\alpha_1$-globulin contained more sialic acid than did the porcine or avian $\alpha_1$-globulins. The porcine $\alpha_2$-globulin contained more than did the other two. Avian $\beta$- and $\gamma$-globulin contained more sialic acid than did those of the other two species.

7. Porcine plasma proteins were separated into fractions
P-1, P-2, P-3, and P-4. The fractions were analyzed for hexose, hexosamine, sialic acid, and fucose. Greatest yields were obtained of the P-1 fraction, smallest of the P-4 fraction. The P-3 and P-4 fractions contained higher percentages of carbohydrate whereas the P-1 and P-2 contained greater overall quantities due to their higher yield.

Fractions were analyzed by cellulose acetate and polyacrylamide gel electrophoresis. The P-1 fraction contained most zones of the whole plasma. The P-2 fraction contained three zones corresponding to albumin, \( \alpha_2 \)- and \( \beta \)-globulin. The P-3 fraction contained three or four zones, of which only one contained appreciable amounts of carbohydrates. The P-4 fraction exhibited one band on cellulose acetate and three on acrylamide gel electropherograms, of which two contained carbohydrate. It was suggested that all four precipitates contained glycoprotein. The P-1 was thought to contain most of those found in the whole plasma while the P-2 and P-3 each contained at least one major glycoprotein and the P-4 at least two.

8. The fractional precipitates were chromatographed on columns of carboxymethyl cellulose, separating into at least three sub-fractions. Sub-fractions were analyzed for characteristic glycoprotein constituents. The P-3-B, P-4-A,
and P-4-B contained the highest percentages of these components. The P-1-B, P-2-B, P-3-A, P-3-B, and P-4-C all contained high amounts when concentrations were expressed on the basis of milligrams per gram of fraction chromatographed. These same fractions, as well as P-4-A and P-4-B were high in hexosamine. Only P-1-C, P-3-B, and P-4-B were high in sialic acid while only P-3-B, P-3-C, and P-4-C were high in fucose.

All fractions were examined by cellulose acetate electrophoresis. All were heterogenous. The P-1 and P-2 fractions as well as the P-3-A, P-3-C, and P-4-C, all exhibited three to five zones. The P-3-B, P-4-A, and P-4-B exhibited two to three zones.

9. The incorporation of glucosamine-1-C\textsuperscript{14} into the serum proteins of White Leghorns was studied following intravenous injection. It was found that incorporation curves varied widely from bird to bird. Maximum incorporation occurred from two to ten hours after injection, after which levels dropped at varying rates.

Electrophoretic distribution of protein, hexosamine and activity were studied following agar electrophoresis. The distributions of hexosamine and activity were not identical. It was suggested that the glycoproteins have different turn
over rates or that all activity was not present as glucosamine-1-C\textsuperscript{14}.
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