AN ABSTRACT OF THE THESIS OF

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Title AN ELECTROPHORETIC ANALYSIS OF THE SERUM PROTEINS IN INFECTIOUS MONONUCLEOSIS

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Thirty-six sera from cases of infectious mononucleosis and 25 normal human serum specimens were studied by electrophoresis on cellulose acetate. The results indicated that:

The mean concentration of total protein in the mononucleosis sera was significantly higher than that of the normal sera. In 15 of the 36 mononucleosis specimens the total protein concentration was above the range of the normals.

The higher protein concentration was due entirely to increased amounts of the globulin fractions. The gamma globulin contributed most to the increase. Its mean concentration was above normal and in 29 of the 36 cases the gamma globulin level was above the range for the normal sera.

With respect to the alpha-2 globulin, the mean concentration was slightly above that for the normals and in nine of the 36 sera the concentration was above the normal range.

The mean concentration of the alpha-1 and beta globulins was slightly higher than the corresponding means for the normals.

Twenty-two mononucleosis sera were studied by electrophoresis on cellulose acetate followed by elution of each separate fraction and testing of the eluates for agglutination of sheep erythrocytes. The gamma globulin fraction of 21 of the 22 specimens agglutinated the sheep cells. The alpha-2 globulin fraction of ten mononucleosis specimens also showed hemagglutination. Nine of these ten sera had an alpha-2 globulin content above the upper limit of normal (0.89 g/100 ml). These results indicated that the heterophile antibodies were present in the gamma globulin fraction in almost all cases and in the alpha-2 globulin in about half the cases.

In experiments in which eight mononucleosis sera were absorbed with beef erythrocytes, complete removal of the heterophile antibodies was observed in four specimens while four others retained a low titer of 1:5. The significant reduction of the gamma globulin fraction in all eight cases and of the alpha-2 fraction in four cases after absorption, with no reduction in other fractions indicates that the heterophile antibodies are mainly in the alpha-2 and gamma globulin fractions of the serum protein. These experiments support the quantitative data of the cellulose acetate experiments and the results of the hemagglutination experiments with the separate protein fractions.

Twelve mononucleosis sera and 12 normal sera were studied by immunoelectrophoresis in agar gel. In all 12 mononucleosis specimens an abnormal pattern was observed in the alpha-2 globulin zone. One and sometimes two of the components showed precipitin arcs longer than in normal sera, extending into the beta-1 globulin zone. These arcs apparently represent one or more altered components of the alpha-2 globulin, appearing on immunoelectrophoretic plates as a population of molecules with a wider than normal range of electrophoretic mobilities. These molecules could very possibly represent the heterophile antibodies shown by other methods to be present in the alpha-2 globulin.

A second observation not revealed by other methods was the fact that in nine of the 12 mononucleosis sera the beta-2M globulin arcs were more heavily stained and more distinct than in the normal sera used for comparison, when antibody against normal and mononucleosis serum was used. Since beta-2M globulin is a recognized antibody carrier, and in view of the observation made by Strannegard (45) that ox cell hemolysins migrated with the beta-2M globulin in mononucleosis sera, the above observation suggests that the increase in the gamma globulin fraction, observed in our electrophoretic analyses of 36 mononucleosis sera, was at least partly due to an increase in the beta-2M globulin, which is not distinguishable from the gamma globulin in electrophoresis on cellulose acetate.

AN ELECTROPHORETIC ANALYSIS OF THE SERUM PROTEINS IN INFECTIOUS MONONUCLEOSIS

by

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

AN ELECTROPHORETIC ANALYSIS OF THE SERUM PROTEINS IN INFECTIOUS MONONUCLEOSIS

INTRODUCTION

Infectious mononucleosis, also called glandular fever, monocytic angina, acute mononucleosis, or Drusenfiber (36, 24)is a disease whose etiological and therapeutic aspects are still in the stage of infancy. The diagnostic aspects were first studied by Paul and Bunnell (37)in 1932. They reported that patients with infectious mononucleosis developed antibodies which agglutinated sheep erythrocytes and were called the sheep cell agglutinins. Since then, the presumptive test of Paul and Bunnell for infectious mononucleosis has been accepted widely for clinical and laboratory diagnosis.

Later, in 1935, Bailey and Raffel (1) discovered that the antibodies found in infectious mononucleosis could also be demonstrated as an hemolytic agent for ox or beef red blood cells. Antibodies exhibiting this behavior were named beef erythrocyte hemolysins. Both the sheep agglutinins and beef hemolysins are as a whole regarded as the heterophile antibodies.

Eyguem (15) reviewed a large number both of his own cases and of those from the literature and contrasted them with normal control groups. He and previous workers (15, 12, 37, 1 and 30) found a significant elevation of the sheep cell agglutinin titer, the ox cell hemolysin titer and the horse cell agglutination titer in patients

with infectious mononucleosis as compared to normal subjects. Agglutinins against rabbit red blood cells were also present in patients with infectious mononucleosis but significant titers were only moderately higher than in normal persons.

Besides the heterophile system of circulating antibodies, antibodies of other types have also been reported in infectious mononucleosis, e. g. antibodies against human red cells treated with Newcastle disease virus (5); the Listeria monocytogenes type antibodies, as described by Stanley (43); the salmonellae type (3); antibodies against red cells sensitized by antigen present in many gram positive cocci (17) and staphylococcal toxin (6) and many others as recorded in the literature. However, these antibodies are not found as consistently as the heterophile antibodies; many of them are found only occasionally, and some of them are poorly authenticated.

Confronted with these complicated observations, we are led to believe that, in infectious mononucleosis, a number of different antibodies are produced which react with antigenic determinants present on a number of heterologous red cells. The question whether these antibodies form part of an immunologic response to the as yet undiscovered infecting agent is of great interest. In view of this complexity, Wilkinson and Carmichael (51), using various immunochemical techniques, and cross absorption and elution experiments, tried to determine whether they were identical macroglobulin

molecules with determinants capable of reacting with a large number of related red cell antigens, or whether, a different group of antibody molecules was reacting specifically with the antigenic determinants on each type of red cells. Their results suggested that the rabbit cell antibodies are separated from the other systems. The Newcastle disease agglutinin is probably also separate. Antibodies against sheep, horse, and ox red blood cells show a well defined pattern of cross absorption, and it is suggested that either these antibodies are separate globulins with similar but non-identical receptors for sheep, horse and ox cells, or that several unrelated antibodies in the sera of patients with infectious mononucleosis react with different and unrelated antigenic determinants shared by heterologous red cells.

Since the sheep cells agglutinins are the type of antibody most constant and pronounced, serving as a diagnostic criterion for the disease being studied, effort was made, in this project, to localize this specific type of heterophile antibody in serum samples of mononucleosis patients. A total of 36 fresh mononucleosis sera from two different sources were analyzed against a control standard set by 25 normal healthy adults whose sera were also analyzed by the same methods.

LITERATURE REVIEW

Detailed information concerning the development of knowledge of heterophile antigen and antibodies, though extremely confusing, was given in Davidsohn's review (9). The term heterophile antibody applies to antibody having the capacity to react with certain antigens which are, in most cases, quite different from and phylogenetically unrelated to the one instrumental in producing the antibody response. There are many heterophile systems but they all contain antigenic substances which are able to stimulate the production of heterophile antibodies. In 1911, Forssman discovered that emulsions of cells obtained from organs of the guinea pig, cat, dog, erythrocytes of sheep and man (blood groups A and AB), and many other mammals including the horse, when injected into an appropriate animal such as a rabbit, are capable of eliciting not only specific but also non-specific antibodies which are demonstrable in varying degree in the form of hemolysins or agglutinins for sheep erythrocytes. Chemically, the substance concerned is defined as a lipoid protein complex. Substances containing these Forssman antigens are widely distributed throughout the animal kingdom and often referred to as the so called guinea pig group of substances. In contrast, stands the so called rabbit group, representing substances in which the Forssman antigen has not been found, such as the organs of the rabbit, pig, ox, rats,

etc. Only a few pathogenic bacteria are known to belong to the guinea pig group and probably for this reason, Forssman antibodies have not been demonstrated in appreciable amounts among patients with diseases induced by bacterial agents. Nevertheless, the phenomenon of heterophile antibody production may occur in human beings into whom substances containing the heterophile antigen have been injected.

Another type of heterophile antigen is the Buchbinder antigen which was found to be distinct from the Forssman antigen and was found in bacteria of the hemorrhagic septicemia group and in erythrocytes of a variety of birds. More recently (7), additional heterophile antigens were described, such as those by Findland and Curnen as shared by human erythrocytes and type 14 pneumoncocci. Still another type described as a lipopolysaccharide from debris of autolyzed rough type I of the pneumoccoci was found by Goebel et al. They all constitute a confusing example of serological complexity.

The presence of heterophile antibody in the human body has been demonstrated in several instances. In the first case, Davidsohn found increased amounts of lysins and agglutinins for beef and sheep red blood cells in the serum of patients who had been injected with horse serum (8). As a corollary to this finding, Davidsohn et al. also made the important observation that the average titers for aggutinins and hemolysins were very much higher among patients

who had been injected with horse serum and who subsequently developed serum illness than among those who did not develop allergic symptoms. The high titer persisted, in some cases, over one year in duration. Also, in normal human serum, the presence of hemolysins and agglutinins for sheep cells in low dilutions has been observed repeatedly (10). It was found that their occurence in normal sera is confined to blood groups A and AB. Davidsohn et al., in their series of studies, have found them to be present in dilutions of 1:5, 1:10 and in two cases, 1:320. Friedberger, et al., in their elaborate investigation of these nonspecific aggutinins in man (18), have determined the presence and concentration of these antibodies in individuals of different age groups. Their results were summarized in Figure 1 using 275 control patients.

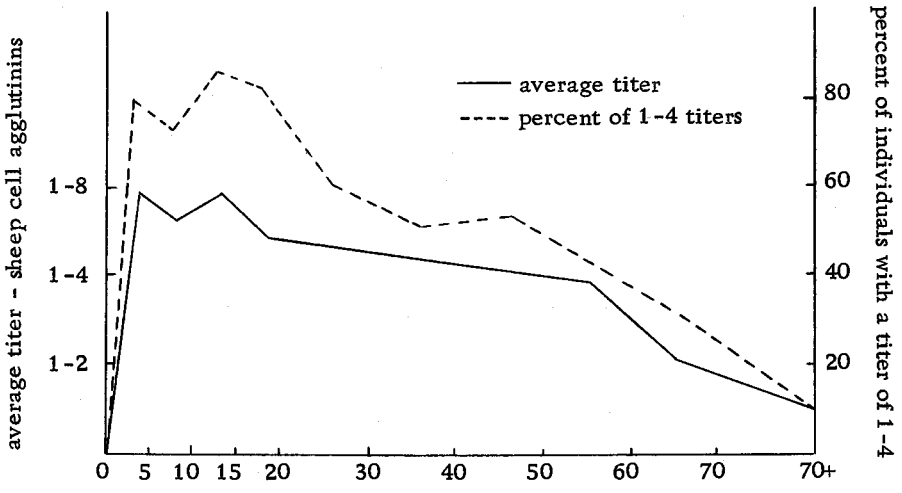

Figure 1. The age distribution of sheep cell agglutinins in sera from a series of 275 general hospital patients.

In interpreting these findings, the German investigators have raised the question to whether defensive antibodies may not be formed in growing children as an expression of immunologic maturation.

Paul and Bunnell, in their pioneer work, associating the presence of heterophile antibodies with the clinical findings in a large number of ailments, accidentally discovered that heterophile antibodies, in the form of sheep erythrocyte agglutinins, were present in very high concentration in the serum of patients in the acute stages of infectious mononucleosis, but not in other diseases that they studied. The workers gave two possible explanations for this finding (37).

1. That the unknown agent responsible for infectious mononucleosis contains the heterophile antigen.

2. That we are dealing with an example of isoagglutinin production elicited by abnormal cells, which are present in the blood, or elsewhere, during active stages of the disease.

The value of the serological test devised by Paul and Bunnell in the diagnosis of infectious mononucleosis has been clearly demonstrated in many papers. The test, as ordinarily applied, however, is not wholly specific, since normal serum and serum from persons who have received horse serum injections, frequently show above normal titers of sheep cell agglutinins. In view of this fact, Davidsohn and coworkers devised an absorption test (13) which was modified

and improved by Stuart (47) employing boiled or untreated beef red blood cells and emulsions of guinea pig kidney, which permits a complete analysis of the type or types of heterophile antibodies under investigation. They found that the complete removal of these antibodies by guinea pig kidney alone indicates a normal serum, because Forssman type antibody is absorbed by guinea pig kidney cells. The absorption of all or most of the antibodies by both beef erythrocytes and guinea pig kidney indicates antibodies of the type present in serum illness which react with both Forssman and non-Forssman antigens, while antibodies present in infectious mononucleosis are absorbed only by beef erythrocytes. The absorption test proved indirectly that heterophile antibodies present in infectious mononucleosis are not the Forssman type since they are not absorbed by the Forssman antigen. This procedure is called the differential diagnosis for infectious mononucleosis and is one of the standard procedures used clinically. An extensive review of this method has been made by Virtanen recently (50).

Very little attention was paid to the behavior and characteristics of these heterophile antibodies in infectious mononucleosis except for the classical observation made by Davidsohn who reported that:

1. Sheep cell agglutinins and beef cell hemolysins increased and decreased in early and late stages of the disease. In the course of the illness, the change in titer of these two antibodies was parallel

to each other.

2. Absorption tests done with beef erythrocytes showed that both sheep cell agglutinins and beef cell hemolysins were completely removed by 5% suspensions of boiled or untreated beef erythrocytes.

3. With the various analytical methods employed they were able to associate these antibodies with the gamma-globulin fractions of the serum proteins.

4. The only difference that they found between these two types of antibodies was that hemolysins were more sensitive to temperature change than sheep cell agglutinins. They concluded that these two were phsyiologically inseparable but may possess different combining sites responsible for agglutination and hemolysis.

In 1963, Davidsohn published another paper with Lee (31) in which they established the conclusion made in the paper of 1935 (13) by introducing new experimental results. They further proved that the agglutinins and hemolysins were not separable by electrophoresis, nor column chromatography and that the sheep cell agglutinins have a higher temperature coefficient. In this paper, Davidsohn recorded a slightly different observation on the beef RBC absorption test. They noticed that the sheep erythrocytes have less ability to remove the beef hemolysins than the red blood cells. To interpret this finding, the author suggested that the beef cells possessed per unit volume a greater amount of the antigens for the sheep cell antibodies

of infectious mononucleosis than did the sheep cells themselves. By using different types of RBC to provide different combination sites, they found that absorption of beef hemolysins by sheep red blood cells was not complete while agglutinins were removed entirely.

The observation of Lee and Davidsohn was supported by Layton (30) in 1952. Layton noticed that beef hemolysins were not absorbed by sheep erythrocytes and opened the question as to the possibility of more than one heterogenetic antigen determinant on ox and sheep RBC responsible for the reactions with infectious mononucleosis serum. Swedish investigators, Orjan Strannegard and Erick Lycke (45), by using agar diffusion immunoelectrophoresis and absorption experiments, also added their view point to this problem. They concluded that:

1. At least two types of antibodies were present in the immune serum of infectious mononucleosis, one reacting with the antigenic factor of the ox cell while the other reacts with the antigenic factor common to both beef and sheep cells.

2. The antibodies precipitating the ox cell antigen were in the beta-2-M globulin fraction.

3. From unpublished results of their associates, they have evidence that these heterophile antibodies were high molecular weight substances, therefore slow moving proteins.

Much has been done on the serological basis of diagnosis for infectious mononucleosis but a review of the medical literature of the past 25 years shows that little attention was given to the localization of these antibodies. Only four investigations were recorded, three of which were done outside of the United States while one was performed by K. Sterling in 1949 (44). Moving boundary electrophoresis was employed for this investigation in which Sterling was able to find that in six out of seven sera from mononucleosis patients, the albumin was diminished and the gamma globulin increased, both in absolute amounts and on a percentage basis, as compared with the normal sera. The only case that did not show such deviations was the non-jaundiced case which gave negative cephalin-cholesterol flocculation and thymol turbidity tests, and in whom the only normal alkaline phosphatase determination was found. Less pronounced and less frequent observed alterations were elevations of the alpha-1-globulin and beta-globulin fractions.

By studying the medical history of these seven patients and their clinical complications besides infectious mononucleosis, Sterling found that liver function tests showed abnormalities in six out of the seven cases. From this he tried to conclude that the alterations of the serum proteins were possibly related to hepatic dysfunction. By absorption with sheep red blood cells of only one serum sample from these patients, Sterling noticed the reduction of the

heterophile titer from 1:2048 to 1:32. The electrophoretic pattern of this serum sample before and after the antibody absorption were found to be identical. From this one sample, the author suggested the possibility that the increase of the gamma globulin was not due to heterophile antibodies.

A German investigator, Frenger (16), by using a hemagglutination method with sheep erythrocytes applied directly on the paper strips used for electrophoretic separation of the serum sample, was able to identify the exact fractions of the serum protein that bore the antibodies. This author used the Paul-Bunnell presumptive test to determine the heterophile titer of infectious mononucleosis serum samples, from which ten with relatively high titer were selected to perform the special agglutination test that he had devised. Electrophoretic separations of these samples were first done on filter paper strips after which a suspension of sheep erythrocytes was spread on the strip to allow agglutination to occur. From this work, he concluded that sera with titers of 1:128 and higher gave satisfactory localization of the hemagglutinating factor, which was shown to be present in the slow migrating portion of the gamma-globulin. In one serum with high sheep cell agglutinin titer, in addition, the author observed some agglutination in the beta region.

The Italian investigators, Giuliani et al. have studied the serum proteins in 34 cases of infectious mononucleosis electrophoretically

(19). The total protein of each sample was determined colorimetrically by the Biuret method. Their findings were tabulated as follows.

	Normal	Increased	Decreased
Total protein	20 cases	8 cases	5 cases
alpha-1 globulin	33	1	0
alpha-2 globulin	0	19	0
beta globulin	25	8	1
gamma globulin	14	20	0
Albumin	16	0	18

The fourth study on the localization of these heterophile antibodies was done by French coworkers, Buri and Eyquem (38). By using immunoelectrophoresis in agar gel, these workers studied 64 serum samples from cases of infectious mononucleosis which gave positive Paul-Bunnell and Davidsohn absorption tests. Among these 64 samples studied, 42 cases showed a definite and pronounced increase in the beta-2M globulin fraction while 12 showed a significant but only moderate increase. These findings suggested, according to the authors, that the antibody responsible for the Paul-Bunnell-Davidsohn reaction must have an electrophoretic mobility corresponding to that of the beta-2M globulin. Only a more precise study using ultracentrifugation would provide confirmation of this hypothesis, said the investigators. However, they offered no explanation for the ten cases where there was no detectable increase in the beta-2M

globulin fraction though the Paul-Bunnell-Davidsohn test was definitely positive.

EXPERIMENTAL MATERIALS AND METHODS

Source of Serum Samples and Method of Preparation

Serum samples for this study were supplied by Dr. R. A. MacHaffie of the Student Health Service of Oregon State University and Dr. G. Brandon of the Oregon Public Health Laboratory, Portland. These were cooled by dry ice during transportation, but not frozen, and sterilized with Swinny type syringe filters using filter pads of pore size 0.22 μ as soon as received. Sterile sera were stored in individual tubes and refrigerated at 4° C.

Normal sera for controls were obtained as pre-natal specimens from healthy adults or from healthy college students. Only those specimens showing total protein values within normal limits of 6.35-7.92 g/100 ml were considered acceptable (Sandford 40, Smith 42, and Kolmer 29).

Mononucleosis sera from Dr. R. MacHaffie were obtained from patients clinically diagnosed as having infectious mononucleosis. The diagnosis was confirmed by the presence of heterophile antibodies which were not removed by guinea pig kidney absorption. Mononucleosis sera from Dr. G. Brandon of Portland were from blood specimens sent in to the Public Health Laboratory by physicians all over the state of Oregon. They were from patients presumably diagnosed clinically by different physicians as infectious

mononucleosis cases and further confirmed by the laboratory procedure of the heterophile antibody titration. These heterophile antibodies were not removed by guinea pig kidney absorption.

Heterophile antibody titration was done on each serum sample used in this study, both normal and mononucleosis, by the method in Kolmer (29), which is the presumptive test of Paul and Bunnell. In this test, the serum to be titrated was first inactivated in a water bath at 56° C for 30 minutes. Then, two-fold serial dilutions of the serum were made with physiological saline, ranging from a 1:5 dilution to 1:5120 or higher if desired. A tube containing saline alone served as the corpuscle control. A 2% suspension of washed sheep erythrocytes was added to each dilution and the mixtures were shaken and left at room temperature for two hours. The results were read after shaking the tubes to resuspend the sediment. The titer was the reciprocal value of the highest dilution of serum that gave visible agglutination to the naked eye or under a low power objective of the microscope. Normal sera with titers above 1:5 and mononucleosis sera with titers below 1:160 were discarded.

The Total Protein Determination

In order to analyze the protein composition of the infectious mononucleosis sera, a total protein determination was done on each sample as a basis for the calculation of the absolute grams of protein

in each serum fraction after they were electrophoretically separated. Instead of analyzing each serum sample for total nitrogen content and converting the nitrogen to protein after the non-protein nitrogen had been subtracted, we used an indirect Biuret method as described by Sandford (40). In this process, a sample of normal serum was first analyzed by the micro Kjeldahl method for total nitrogen content. The sample was then analyzed for non-protein nitrogen as described by Kolmer (29, Kolmer, p. 977). The total protein nitrogen of the serum was obtained by subtracting the non-protein nitrogen from the total nitrogen and the remainder was converted to total protein by multiplying by a factor of 6.54. According to Sunderman, human serum protein contains 15.3% nitrogen which is slightly lower than the traditional assumption of 16% (40, Sandford, p. 435) (49).

In order to analyze a standard human serum, we first submitted this serum to the micro Kjeldahl to determine its total nitrogen content. This was done by Mr. John Gilmour in the Department of Soils. He analyzed four 2.0 ml replicates of serum sample with the following results:

aliquot No.	1	--	23.36 mg N_2
	2	--	23.60 mg N_2
	3	--	23.44 mg N_2
	4	--	23.48 mg N_2
	average		23.47 mg N_2/2.0 ml of serum

The mean value then indicated 11.735 mg N_2/ml = 1173.5 mg N_2/100 ml of serum.

The non-protein nitrogen determination was also done on four replicate samples of the same serum giving an average content of 22.7 mg N_2/100 ml of serum. From this, it was calculated that the standard serum contains:

1173.5 - 22.7 mg N_2/100 ml

=1150.8 mg of protein N_2/100 ml of serum

To convert N_2 to protein using Sunderman's factor:

1150.8 x 6.54

=7.52 g/100 ml

Therefore, the standard serum contains 7.52 g of protein/100 ml.

This analyzed normal serum sample, with a known quantity of protein, was used as the standard serum with which all the other normal and nononucleosis sera were compared colorimetrically for the total protein determination. It was preserved for use during this entire study by storing in individual tubes in -60° C freezer.

The Biuret method itself involved the addition of 1.9 ml of 0.9% NaCl to 0.1 ml of the serum to be tested. The standard serum was also treated the same way. A blank of 2.0 ml of NaCl solution alone was prepared. Four ml of Biuret reagent (see Solutions and Reagents) was added to each of the mixtures above. In order to

remove any turbidity due to the presence of lipids, 2 ml of ether was added to each and the tubes were shaken vigorously and centrifuged. The ether layer was removed with a pipette. The serum mixture was transferred to a cuvette and its optical density measured in a Beckman B spectrophotometer at a wave length of 580 mμ using the blank to set a zero absorbance. Both the standard serum and the unknown were read for their optical densities. Since the standard serum had been analyzed for its total protein content the protein in unknown sample was calculated by the formula:

$$\frac{\text{Protein Concentration of Standard}}{\text{O. D of Standard}} \times \text{O. D. of Unknown} = \text{Protein Concentration of Unknown}$$

The following example serves to illustrate the calculation of a total protein determination:

Tube Content	Optical Density
Blank	0.000
Standard Serum	0.315
Unknown Serum in Triplicate	
No. 1	0.302
No. 2	0.300
No. 3	0.301
average	0.301

$$\text{Protein Concentration of Unknown} = \frac{7.52 \times 0.301}{0.315}$$

$$= 7.18 \text{ g/100 ml}$$

The Biuret reaction for the estimation of protein in urine was first introduced by Riegler, Autenreith and Hiller. Fine was the one who modified and improved the method (25). They all agreed that the Biuret reaction was reliable and that the error usually did not exceed 5% in experienced hands. According to Kingsley and Robinson (25), who further modified and improved the method for determination of serum protein, blood serum proteins form Biuret color solutions which by analysis on the spectrophotometer, give optical densities (at 580 mμ) that bear a linear relationship with the protein nitrogen concentration as determined by Kjeldahl procedure. The optical density values remain practically constant for at least 48 hours. Results of this method show satisfactory agreement with those obtained from Kjeldahl determination.

One of the difficulties in the quantitative determination of proteins lies in the qualitative changes in the proteins that occur in disease. If a new protein appears that has a different nitrogen or tyrosine content, methods based on these components will give results that are affected by this qualitative change and may not indicate only the change in amount of protein. However, the Biuret reaction uses an alkaline copper sulfate reagent. It depends on the peptide links that characterize protein and appears to be very little affected by qualitative changes in the protein. Therefore the use of this procedure is warranted for the reliable estimation of serum protein when

the amount of sample, time and temperature are limited (40).

Electrophoretic Analysis

All serum samples, both normal and mononucleosis, were subjected to electrophoretic analysis using cellulose acetate as the supporting medium. The apparatus for zone electrophoresis, methods and procedures have been fully described by Kohn (27) and Smith (42).

Zone electrophoresis has chiefly been applied to the separation of protein fractions especially those of serum protein. It is a simple method which provides sharp separation under mild conditions suitable to materials such as proteins which are quite susceptible to alterations. In recent years, such supports as starch or agar gel have partially displaced filter paper because they give much clearer and more definite separations, and better resolution. In 1957, J. Kohn described the use of cellulose acetate as a supporting medium for zone electrophoresis (27) which combines many of the advantages of paper and gel (42).

The reason that the cellulose acetate technique is called micro electrophoresis is that the sample used is small (5 μl). The regularity of structure of the pores in this material allows regular and orderly diffusion. Excellent results with immuno-diffusion and immuno-electrophoretic methods can be obtained with the least

spreading and disturbance. In our work, we found that albumin tailing which is the main problem of zone electrophoresis, is almost eliminated. Another fine feature of cellulose acetate that we found is the perfect reproducibility of the electrophoretic pattern.

The universal horizontal type of electrophoretic apparatus was used in our work (Smith, p. 58-61). The apparatus was designed in such a way that the buffer compartments occupy the whole width and length of the tank to provide a continuous fluid surface, to reduce convection currents and to give a better cooling system by providing more efficient vapour saturation.

For our work, cellulose acetate strips 2. 5 cm wide and 20 cm long were used. They were preferred to the larger strips because the separations were as good but only one-third of the time was required. Detailed discussions on the treatment of the strips and application are given in Smith (42, p. 62-90). After the strips were properly applied onto the holders, the serum sample was streaked on the strip as a straight line. The sample was delivered by means of a capillary tube which was calibrated to give approximately 5 µl of serum per strip. The capillary outflow was controlled by blowing gently with the mouth. With experience, this action could be controlled to such an extent that a gentle blow would give a smooth and straight streak with the help of a ruler.

The position of the starting line influences and determines the

pattern of the separation. By trial and error, we have found that the best results were obtained when the sample was applied about one quarter distance of the bridge gap from the cathode end.

For serum protein electrophoresis, a barbitone buffer of pH 8. 6 was used. In our work, we employed buffers of different molarities for impregnation of the strips and filling of the electrophoretic tank (see Solutions and Reagents).

The electric current supply for the actual run followed the rule of 0. 5 mA/cm of strip width with a gap 8 cm wide. The method of constant current was chosen because:

$$\text{Current} = \frac{\text{Voltage}}{\text{Resistance}}$$

During fluctuation of the resistance, a flexible voltage will automatically adjust to a constant current which will give a more constant speed of migration and at the same time, prevent excessive evaporation from the strips. The optimal time for the electrophoretic run depends on many factors: the freshness of the buffer solution, the room temperature, and the amount of sample used. An average of two and one-half hours was usually sufficient to give satisfactory separation of the bands.

After the electrophoretic run was completed, the strips were stained with a 0. 2% aqueous Ponceau S solution in 3% trichloroacetic acid as a protein coagulant. After five minutes staining, the strips

were rinsed in a 5% acetic acid solution to remove the excess stain and to clear the background.

Different authors have emphasized different techniques of drying the strips. The author has found, through the course of this work, that room temperature over night gave the best drying results because the bands were not distorted as when artificial heat was used. It was also found that the bands appeared sharper when wet. Cutting of the bands when they were still wet and drying them in individual tubes gave a more accurate result during the elution procedure that followed.

The Quantitative Determination of Protein Fractions

Another fine feature of the cellulose acetate strip is its minimal adsorption and relatively homogenous composition which enable the separated protein bands to be eluted for quantitative analysis.

In our work, after the stained strips were thoroughly washed free of excess dye, they were cut into individual protein bands and each band placed in a small serological tube. Each protein fraction, having been precipitated by the Ponceau S dye in trichloroacetic acid, was then eluted by addition of 5.0 ml of 1/10 N NaOH solution. Thirty minutes were usually allowed for the elution period, the end point of which could be judged by the complete removal of the dye from the strip when the tube was shaken. To the eluate was then added

0. 1 ml of 40% acetic acid for each ml of NaOH used. This acidification procedure was to intensify the color to be read on the spectrophotometer at a wave length of 540 mμ. A control blank was prepared by eluting a similar portion of unstained strip, containing no protein, with NaOH and acidifying with acetic acid. From the optical density of each eluted protein fraction, the percent composition of each fraction was determined. By using the total protein content of that specific serum, the absolute protein content of each fraction in grams per 100 ml was calculated. Since both normal and mononucleosis sera were analyzed in the same way, it was possible to study any deviation from the normal pattern. Table No. 1 gives a brief example of an analysis of a normal and a mononucleosis serum, including the quantitative determination of the absolute grams of protein per 100 ml of serum for each electrophoretic fraction of each sample.

Localization of the Hemagglutinating Antibody on the Electrophoretic Strips

The German investigator, Franger (16), in his studies with infectious mononucleosis sera, was able to localize the heterophile antibodies in electrophoretically separated serum using filter paper as the supporting medium. In his experiments, he employed a two to three times larger volume of serum than ordinarily required for

Table 1. Comparison of a mononucleosis and normal serum after electrophoretic analysis.

Type of Specimen	Protein Fractions															Total Protein
	Albumin			α_1 globulin			α_2 globulin			β globulin			γ globulin			
	O. D.[1]	%[2]	gm.[3]	O. D.	%	gm.	O. D.	%	gm.	O. D.	%	gm.	O. D.	%	gm.	
Normal serum	0. 550	63. 65	4. 174	0. 025	2. 89	0. 189	0. 083	9. 60	0. 629	0. 090	10. 41	0. 683	0. 116	13. 42	0. 880	6. 557 g/100 ml
Mononucleosis serum	0. 255	50. 90	3. 863	0. 012	2. 40	0. 182	0. 080	15. 97	1. 212	0. 047	9. 38	0. 712	0. 107	21. 36	1. 621	7. 590 g/100 ml

1 O. D. means optical density.

2 % is calculated as percent of total protein.

3 gm. is the absolute gram of protein/100 ml of serum.

electrophoresis. After the separation was completed, a 6% suspension of washed sheep erythrocytes was placed evenly on the electrophoretic strip which had been again moistened by means of a fine pipette. Since sheep erythrocytes contain the heterophile antigen, they were agglutinated up to certain dilutions by serum specimens containing the specific antibody in sufficient concentration. Retention of the agglutinated red cells in a particular protein band on the strip revealed the location of the antibodies.

In adapting this technique to electrophoresis on cellulose acetate strips, we have modified Franger's method which did not give satisfactory results on the medium that we used. Briefly stated, the modified procedure was as follows: a 10 μl serum sample was employed for each strip in electrophoretic analysis. After the separation was completed, a small portion of each strip was stained and washed to show the location of each protein fraction while the remaining major portion of each strip was cut into individual bands which were put into individual serological tubes. One ml of physiological saline was pipetted into each of the five tubes containing the five separated bands and to a number six tube serving as corpuscle control. The protein fractions were eluted for 30 minutes by suspending in a saline solution, after which, a 0.5 ml of a freshly washed 6% sheep erythrocyte suspension, which was prepared on the same day, was added to each tube. The tubes were shaken, incubated at room temperature for two

hours and the agglutination read after resuspending the sediment. Readings were made with the naked eye or by the low power objective of the microscope (10x).

Absorption of Serum Specimens with Beef Erythrocytes

The differential test for infectious mononucleosis, which is based on the fact that the sheep cell agglutinins and ox cell hemolysins are both absorbed by beef erythrocytes but not significantly by guinea pig kidney, has permitted the differentiation of this disease from other diseases such as serum sickness in which there is also an increase in heterophile antibodies which, however, are largely absorbed by guinea pig kidney (47). Stuart et al. noticed a parallel reduction of the agglutinins and hemolysins in every infectious mononucleosis serum absorbed with beef erythrocytes. However, they suggested that for diagnostic purposes, it is more satisfactory to work with agglutinins because variation from the average normal titer appears to be far less with this type of antibody (11).

In our investigation, absorption tests were performed on eight mononucleosis specimens in order further to support our observations in the electrophoretic analysis. For sera to be absorbed by beef erythrocytes, we first obtained the electrophoretic pattern of each before submitting them to absorption. A detailed description of the absorption procedure used is given in Simmons and Geutzkow (41, p. 864). Briefly, in their method, a 0.5 ml volume of packed beef erythrocytes, which had been washed three times with physiological

saline, was added to 1.0 ml of a 1:2 dilution of the serum to be tested. Cells and serum was mixed and incubated at 37° C for 30 minutes. However, we modified the absorption time and temperature by placing the mixture in a 4° C refrigerator overnight and found this procedure gave us more or less complete absorption of antibody without repeating the process. The mixture was then centrifuged at 4000 rpm for 20 minutes to remove the red blood cells. A portion of this supernatant was used for heterophile antibody titration with sheep erythrocytes (29, Kolmer p. 789). Absorbed samples showing no or very low heterophile titers were subjected to another electrophoretic analysis to compare the protein fractions with those present before absorption. Since each serum sample was diluted before absorption, reconstituting to its original solid content was necessary before the second electrophoretic run. This was done by drying 0.1 ml of each original serum sample in a desiccator with P_2O_5 before absorption was to take place. The total solid per unit volume of serum served as a guide to reconstitute the serum with distilled water after absorption was completed. Then the serum was subjected to the second electrophoretic run.

Immunoelectrophoresis

Immunoelectrophoresis is another tool that we used to study our problem. It is a very sensitive method by which additional fractions of immunologically recognizable protein having the same or similar

mobilities can be separated as different precipitin bands or arcs through reaction with antiserum.

This method was first introduced by Grabar and Williams (70) and others (22, 23). By combining the electrophoretic separation with an immunological reaction in gel, these workers have been able to show the existence of more than 20 components in human serum by virtue of differences in their electrophoretic characteristics and diffusion rates and by their precipitin reactions with anti-human serum. This implies that electrophoretically identical but immunologically different substances can be demonstrated, and likewise, the immunological relationship between electrophoretically non-identical components can also be studied.

In this method (22), the specimen to be studied is first submitted to electrophoresis in agar gel or cellulose acetate as the supporting medium. At the end of the run, when the protein components are separated according to relative mobilities, immuno-precipitation takes place between the antigens in the specimen (e. g. mononucleosis serum) and antibodies specific for these antigens (e. g. immune rabbit serum against infectious mononucleosis serum) which is applied along a line parallel and close to the separated pattern. For each antigen-antibody system, a distinct line or arc of precipitate will form in the supporting medium. Each immuno-precipitate functions as a selective barrier to the antigen-antibody system that gives rise to it but does not prevent other immunologically unrelated antigens or

antibodies from passing. Moreover, the distance of this immunoprecipitate from the two lines of diffusion depends upon both the concentration and the diffusibility of the antigen and antibody giving rise to this precipitate. For this reason the probability that the different antigen-antibody reactions will appear as distinct, non-superimposed precipitates in the gel or other supporting medium is high.

In principle, an immunoelectrophoretic analysis is carried out in two parts. The first stage comprises an electrophoretic separation under conditions analogous to those, for example, in paper or zone electrophoresis, but where the stabilizing medium is a mixture of buffer and agar poured on a microscopic slide. The equipment used for this work was the LKB apparatus for agar gel electrophoresis. For making up the agar medium and filling the electrophoretic tanks, a barbitone buffer of pH 8.2 and ionic strength of 0.1 was used (see Solutions and Reagents). For preparation of the gel-plated slides, one part of Difco Noble agar, 25 parts of buffer and 75 parts of distilled water were mixed, and dissolved in a boiling water bath. To prevent the growth of bacteria, merthiolate to a final concentration of 1:10,000 was added.

This mixture of melted agar and buffer was poured onto 26 mm x 76 mm microscopic slides which had first been cleaned with 2% potassium bichromate in concentrated sulfuric acid, thoroughly rinsed in distilled water, and then stored in 95% ethanol until required for

use. The slides were first impregnated in a special agar glycerin solution (see Solutions and Reagents) to improve the adhesion of the agar to the slide surface. The slides were placed in a slide frame which holds three slides in each of two sections. Ten ml of the hot buffered agar were poured on each section of the frame which was supported by a levelling table. After the agar was set and cooled, the slides were placed in a humid chamber for 30 minutes. Just before using the agar-covered slides, two wells, 1.5 mm in diameter, were punched in the agar on each slide in the positions shown in Figure 2, using a special punch. A trough of 2.0 mm in width and 60 mm in length, as shown in Figure 2, was made at the same time. Excess agar was sucked away from each well with a fine tipped pipette, taking care not to damage the wells. With a curved micro pipette, 0.2 µl of one of the mononucleosis sera was pipetted into one well. The same volume of a normal serum specimen was placed in the opposite well. Both were subjected to electrophoresis for one hour with a current of 50 milliamperes and a potential of 250 volts. After the electrophoretic run, antiserum was added to each trough according to the pattern of analysis desired. This is the second stage of the method. The exact volume of antiserum was not critical because this system requires antibody in excess. Figure 2 shows the type of arrangement that we used in our work. All serum specimens were prepared in duplicate slides. One of these was treated with

antiserum against normal human serum. The other was treated with antiserum against mononucleosis serum.

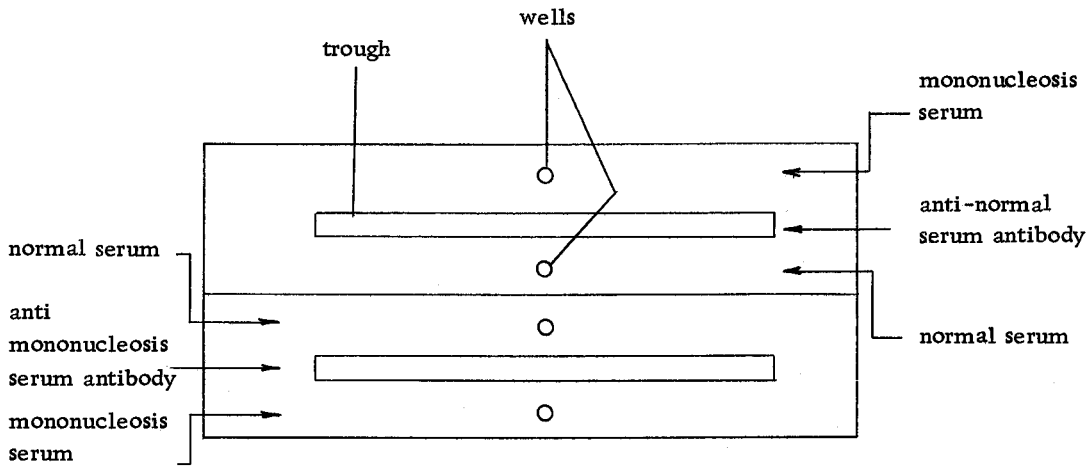

Figure 2. Diagram showing arrangement of serum and antiserum on immunoelectrophoretic plates.

The immunochemical reactions were allowed to take place in a humidity chamber saturated with water vapor for 20 hours. At the end of this time, the slides, kept in frames and locked in a holder, were put into the following solutions in the proper order.

1. 1% NaCl solution for six hours to remove the excess proteins.
2. 1% NaCl solution for another 16 hours.
3. Distilled water for one hour.
4. The slides were dried in a 35° C oven until dry.
5. Into amido black staining solution (see Solutions and Reagents) for five minutes.

6. Into first rinsing solution for ten minutes (see Solutions and Reagents).

7. Into second rinsing solution for ten minutes.

8. Into third rinsing solution for ten minutes.

9. Into fourth rinsing solution for ten minutes.

After step 9, the slides were carefully removed from the frames, air dried and labelled.

Antibody required for this technique was produced by injecting rabbits with infectious mononucleosis serum or with normal human serum prepared by the method of Proom (39). The antigen was prepared by pooling small samples of the serum to give sufficient quantity for subsequent injections of the rabbits. In the case of the mononucleosis serum, a total of 20 different sera from the Oregon State Public Health Laboratory were pooled. In the case of the normal serum, a total of 21 different sera from the same source were pooled. The heterophile titer of each mononucleosis and normal serum was determined before pooling. The mononucleosis sera gave titers ranging from 1:160 to 1:5120 while the normal sera had only one sample giving a titer of 1:5. Twenty-five ml of each type of serum were added to 80 ml of sterile distilled water. A solution of 90 ml of sterile 10% potassium alum was added to the serum mixture. The pH was adjusted to 6.5 with 5 N NaOH and the mixture centrifuged. The precipitate was washed twice with 200 ml of 1:10,000 merthiolate in

physiological saline and the final precipitate made to a volume of 100 ml with the same solution. The final mixture was tested in thioglycolate medium for sterility.

Ten ml of this antigen were equivalent to 2. 5 ml of the original serum. Proom found that intramuscular injection of 5 ml of the antigen into both hind legs of the rabbit according to the following schedule will produce higher potency and more specific antibody than multiple injections by the intraperitoneal or intravenous routes. The rabbits were also found to tolerate this procedure better. We used the same schedule. Two rabbits received the prepared mononucleosis serum, and two received the normal serum.

Injection schedule for rabbits:

1st day - Inject 5 ml treated serum intramuscularly into each buttock

14th day - Inject 5 ml treated serum intramuscularly into each buttock

24th day - Inject 1 ml untreated serum intraperitoneally

34th day - Collect approximately 50 ml blood from each rabbit.

The immune sera were collected on the 34th day after the first injection. Clots were removed carefully and the sera refrigerated over night. They were then centrifuged to remove the red cells, sterilized by Seitz filtration and stored at 4° C.

Solutions and Reagents

Biuret Reagent for Total Protein Determination

Prepare concentrated carbonate free NaOH and determine its concentration as described in Sandford (40, p. 399). Calculate the volume of this solution that contains 69 grams of NaOH and measure this volume into a 500 ml graduated cylinder. Add water to the 300 ml mark. Add 100 ml of 1% copper sulfate pentahydrate. Mix well. The solution should be clear.

Buffer Solutions for Cellulose Acetate Electrophoresis

For serum and plasma electrophoresis, a barbitone buffer, pH 8. 6 is recommended.

For impregnation of the strip: 0. 07 M

Barbitone	-----	1. 66 gm
Sodium diethyl barbitone	-----	12. 76 gm
Distilled water to one liter		

For filling the electrophoretic tank: 0. 06 M

1000 ml of the above buffer plus 20 ml distilled water.

Barbitone Buffer for Immunoelectrophoresis, pH 8. 2, Ionicity 0. 1

15. 85 gm Sodium barbital
770 ml distilled water
230 ml 0. 1 N HCl

Impregnation Solution for Immuno Electrophoresis Slides

0.1% agar (Difco Noble agar), 0.05% glycerin, in distilled water.

Amido Black Staining Solution

To 1500 ml of rinsing solution (see below) add nine gram of amido black 10 B.

Rinsing Solution for Immuno Electrophoresis Slides

450 ml of methyl alcohol
100 ml of glacial acetic acid
450 ml of distilled water

to make a liter of solution.

Statistical Methods

In our electrophoretic analysis on cellulose acetate for each sample analyzed, we took the mean value of four replicate strips to reduce the accidental error of the method. On account of the variation between results from the four strips and also the difference between the means of each sample, it was necessary to determine statistically the unavoidable error in an analysis and the least significant difference between the mean values from the four strips.

Procedures used in determining these statistics are fully described in Mainland (33, p. 159 and 204).

Twenty-five sets of four replicate values obtained from electrophoresis of 25 mononucleosis sera were used for such an analysis. The alpha-1 globulin, representing the smallest fraction and the gamma globulin, the largest globulin fraction and the one that is of most interest, were selected for this purpose. The data and calculations for the alpha-1 globulin are shown in Table 2 while those for the gamma globulin are shown in Table 3.

Table 2. Statistical analysis of the alpha-1 globulin from four replicate strips.

Mono-nucleosis	a_1 globulin from 4 replicate strips (g/100 ml)				Mean value of 4 strips	Deviation from mean				Square of deviation from mean				Sum of squares of
Serum	1	2	3	4	Mean	1	2	3	4	1	2	3	4	4 dev.
1	0.182	0.374	0.405	0.331	0.323	0.141	0.051	0.082	0.008	0.019881	0.002601	0.006724	0.000064	0.029270
2	0.299	0.305	0.327	0.298	0.307	0.008	0.002	0.020	0.009	0.000064	0.000004	0.000400	0.000081	0.000549
3	0.372	0.270	0.260	0.187	0.272	0.100	0.002	0.012	0.085	0.010000	0.000004	0.000144	0.007225	0.017373
4	0.319	0.333	0.455	0.390	0.374	0.055	0.041	0.081	0.016	0.003025	0.001681	0.006561	0.000256	0.011523
5	0.413	0.437	0.457	0.487	0.449	0.036	0.012	0.008	0.038	0.001296	0.000144	0.000064	0.001444	0.002948
6	0.277	0.347	0.344	0.375	0.336	0.059	0.011	0.008	0.039	0.003481	0.000121	0.000064	0.001521	0.005187
7	0.230	0.305	0.260	0.322	0.279	0.049	0.026	0.019	0.043	0.002401	0.000676	0.000361	0.001849	0.005287
8	0.356	0.426	0.378	0.420	0.395	0.039	0.031	0.017	0.025	0.001521	0.000961	0.000289	0.000625	0.003396
9	0.368	0.434	0.406	0.453	0.415	0.047	0.019	0.009	0.038	0.002209	0.000361	0.000081	0.001444	0.004095
10	0.297	0.320	0.331	0.319	0.317	0.020	0.003	0.014	0.002	0.000400	0.000009	0.000196	0.000004	0.000609
11	0.337	0.349	0.386	0.371	0.361	0.024	0.012	0.025	0.010	0.000576	0.000144	0.000625	0.000100	0.001445
12	0.266	0.195	0.245	0.305	0.253	0.013	0.058	0.008	0.052	0.000169	0.003364	0.000064	0.002704	0.006301
13	0.383	0.391	0.406	0.258	0.360	0.023	0.031	0.046	0.102	0.000529	0.000961	0.002116	0.010404	0.014010
14	0.396	0.487	0.414	0.350	0.412	0.016	0.075	0.002	0.062	0.000256	0.005625	0.000004	0.003844	0.009729
15	0.372	0.324	0.329	0.378	0.351	0.021	0.027	0.022	0.027	0.000441	0.000729	0.000484	0.000729	0.002383
16	0.373	0.311	0.364	0.348	0.349	0.024	0.038	0.015	0.001	0.000576	0.001444	0.000225	0.000001	0.002246
17	0.221	0.292	0.389	0.284	0.297	0.076	0.005	0.092	0.013	0.005776	0.000025	0.008464	0.000169	0.014434
18	0.395	0.402	0.365	0.438	0.400	0.005	0.002	0.035	0.038	0.000025	0.000004	0.001225	0.001444	0.002698
19	0.332	0.283	0.312	0.238	0.291	0.041	0.008	0.021	0.053	0.001681	0.000064	0.000441	0.002809	0.004995
20	0.497	0.343	0.332	0.410	0.396	0.101	0.053	0.064	0.014	0.010201	0.002809	0.004096	0.000196	0.017302
21	0.401	0.368	0.429	0.416	0.404	0.003	0.036	0.025	0.012	0.000009	0.001296	0.000625	0.000144	0.002074
22	0.569	0.433	0.393	0.371	0.441	0.128	0.008	0.048	0.070	0.016400	0.000064	0.002304	0.004900	0.023668
23	0.286	0.370	0.294	0.440	0.347	0.061	0.023	0.053	0.093	0.003721	0.000529	0.002809	0.008649	0.015708
24	0.281	0.341	0.309	0.316	0.312	0.031	0.029	0.003	0.004	0.000961	0.000841	0.000009	0.000016	0.001827
25	0.520	0.550	0.461	0.514	0.511	0.009	0.039	0.050	0.003	0.000081	0.001521	0.002500	0.000009	0.004111
												Total sum of squares		0.203168
														$= 20.3168 \times 10^{-2}$

Table 2 Continued

Total sum of square of deviation = 20.3168×10^{-2} ①

Degrees of freedom = $(4-1) \times 25$
$= 75$ ②

$$\underline{\text{Variance}} = \frac{①}{②} = \frac{20.3168 \times 10^{-2}}{75}$$

$$= 27.089 \times 10^{-4}$$

Estimated standard deviation

$$= \pm \sqrt{\text{Variance}}$$

$$= \pm \sqrt{27.089 \times 10^{-4}}$$

$$= \pm 5.205 \times 10^{-2}$$

This means that when 4 replicate strips are run and the mean value taken, the true value for the α_1 globulin is unlikely to be above or below the mean value found by more than 2×0.05205 or 0.1041 g/100 ml of serum.

Standard error of difference between means of 4 strips

$$= \pm \sqrt{\frac{(0.05205)^2}{\sqrt{4}} \times 2}$$

$$= \frac{0.05205}{2} \sqrt{2}$$

$$= 0.036792$$

In Fisher's table of t with 75
$p = 0.05, \quad t = 2.0$

The least significant difference must be $> 2 \times 0.036792$
$= 0.073584$ g/100 ml of serum.

Hence, if 2 serum specimens are analyzed for α_1 globulin, the results must differ by more than 0.074 g/100 ml before the difference can be considered significant.

Table 3. Statistical analysis of the gamma globulin from four replicate strips.

Mono-nucleosis	γ globulin from 4 replicate strips (g/100 ml)				Mean value of 4 strips	Deviation from mean				Square of deviation from mean				Sum of squares of
Serum	1	2	3	4	Mean	1	2	3	4	1	2	3	4	4 deviation
1	1.621	1.678	1.680	1.518	1.624	0.003	0.054	0.056	0.106	0.000009	0.002916	0.003136	0.011236	0.017297
2	1.369	1.384	1.434	1.450	1.409	0.040	0.025	0.025	0.041	0.001600	0.000625	0.000625	0.001681	0.004531
3	1.477	1.549	1.836	1.369	1.558	0.081	0.009	0.278	0.189	0.006561	0.000081	0.077284	0.035721	0.119647
4	0.968	1.017	0.910	0.948	0.961	0.007	0.056	0.051	0.013	0.000049	0.003136	0.002601	0.000169	0.005955
5	1.289	1.190	1.178	1.251	1.227	0.062	0.037	0.049	0.024	0.003844	0.001369	0.002401	0.000576	0.008190
6	1.098	1.125	1.061	1.008	1.073	0.025	0.052	0.012	0.065	0.000625	0.002704	0.000144	0.004225	0.007698
7	1.287	1.321	1.219	1.238	1.266	0.021	0.055	0.047	0.028	0.000441	0.003025	0.002209	0.000784	0.006429
8	1.468	1.380	1.442	1.479	1.442	0.026	0.062	0.---	0.037	0.000176	0.003844	0.------	0.001369	0.005889
9	0.976	0.899	0.937	0.866	0.920	0.056	0.021	0.017	0.054	0.003136	0.000441	0.000289	0.002916	0.006782
10	0.910	0.895	0.876	0.873	0.889	0.021	0.006	0.013	0.016	0.000441	0.000036	0.000169	0.000256	0.000902
11	1.534	1.493	1.545	1.600	1.543	0.009	0.050	0.002	0.057	0.000081	0.002500	0.000004	0.003249	0.005834
12	1.919	1.927	1.848	1.888	1.896	0.023	0.031	0.048	0.008	0.000529	0.000961	0.002304	0.000064	0.003858
13	1.462	1.540	1.587	1.561	1.538	0.076	0.002	0.049	0.023	0.005776	0.000004	0.002401	0.000529	0.008710
14	2.140	2.195	2.169	2.204	2.177	0.037	0.018	0.008	0.027	0.001369	0.000324	0.000064	0.000729	0.002486
15	1.220	1.273	1.235	1.220	1.237	0.017	0.036	0.002	0.017	0.000289	0.001296	0.000004	0.000289	0.001878
16	1.930	1.875	1.912	1.991	1.927	0.003	0.052	0.015	0.064	0.000009	0.002704	0.000225	0.004096	0.007034
17	1.403	1.224	1.278	1.316	1.305	0.098	0.081	0.027	0.011	0.009604	0.006561	0.000729	0.000121	0.017015
18	1.424	1.567	1.404	1.670	1.516	0.092	0.051	0.112	0.154	0.008464	0.002601	0.012544	0.023716	0.047325
19	1.717	1.615	1.593	1.671	1.649	0.068	0.034	0.056	0.022	0.004626	0.001156	0.003136	0.000484	0.009400
20	1.263	1.251	1.233	1.467	1.304	0.041	0.053	0.071	0.163	0.001681	0.002809	0.005041	0.026569	0.036100
21	0.967	1.013	0.941	1.010	0.983	0.016	0.030	0.042	0.027	0.000256	0.000900	0.001764	0.000729	0.003649
22	1.498	1.429	1.509	1.443	1.470	0.028	0.041	0.039	0.027	0.000784	0.001681	0.001521	0.000729	0.004715
23	1.285	1.265	1.273	1.226	1.262	0.023	0.003	0.011	0.036	0.000529	0.000009	0.000121	0.001296	0.001955
24	1.573	1.556	1.527	1.478	1.534	0.039	0.022	0.007	0.056	0.001521	0.000484	0.000049	0.003136	0.005190
25	1.631	1.636	1.760	1.637	1.666	0.035	0.030	0.094	0.027	0.001225	0.000900	0.008836	0.000729	0.011690
													Total sum of squares =	0.350159
														$= 35.0159 \times 10^{-2}$

Table 3 Continued

Total sum of square of deviation = 35.0159×10^{-2}

Degrees of freedom = $(4-1) \times 25$
$= 75$

Variance = $\frac{35.0159 \times 10^{-2}}{75}$

$= 46.688 \times 10^{-4}$

Estimated standard deviation = $\pm \sqrt{46.688 \times 10^{-4}}$

$= \pm \quad 6.835 \times 10^{-2}$

Thus the true value for a γ globulin analysis is unlikely to be above or below the mean value obtained from 4 strips by more than 2×0.0684 or 0.1368 g/100 ml of serum.

Standard error of difference between means of 4 strips

$$= \sqrt{\left(\frac{0.06835}{\sqrt{4}}\right)^2} \times 2$$

$$= \frac{0.06835}{2} \quad \sqrt{2}$$

$$= 0.034175 \times 1.414$$

$$= 0.048323$$

As in the α_1 analysis, $t = 2$
Then the least significant difference must be
$> 2 \times 0.048323$
or > 0.09665 g/100 ml serum.

That is, 2 specimens must differ in γ globulin by more than 0.0967 g/100 ml before the difference can be considered significant.

EXPERIMENTAL RESULTS

Electrophoretic Analysis of Mononucleosis and Normal Sera on Cellulose Acetate Strips

Electrophoretic analysis was the first experimental procedure that we used to obtain a quantitative picture of the protein content of each separated fraction of mononucleosis serum specimens. In doing this, we were interested in determining whether any quantitative changes could be detected in the electrophoretic fractions of sera from cases of infectious mononucleosis; and to examine the possible relationship of any changes found to the presence of the heterophile antibodies.

In this part of the work, a total of 61 serum samples were analyzed electrophoretically on cellulose acetate strips, as described in the section on Materials and Methods. The total protein content of each serum was determined by the Biuret method, using standard serum whose protein content was determined by the micro Kjeldahl method. Each separated and stained serum fraction was eluted from the strip and the optical density of the eluate was measured. The percent composition and the grams of protein/100 ml for each fraction was calculated.

Among these 61 serum samples, 36 were mononucleosis sera and 25 were normal sera. The 36 mononucleosis sera were obtained

from two different sources. Twenty-five of them were from the Oregon State Public Health Laboratory in Portland. They were blood samples sent into the Public Health Laboratory by physicians all over the State of Oregon from patients presumably diagnosed clinically by different physicians as infectious mononucleosis cases and further confirmed by the Paul-Bunnell procedure of heterophile antibody titration. These heterophile antibodies were not removed by the differential test of guinea pig kidney absorption. The heterophile antibody titers for these sera are shown in Table 5. The remaining 11 samples of the mononucleosis group were supplied by Dr. R. A. MacHaffie of the Student Health Service of Oregon State University. These were from patients clinically diagnosed as having infectious mononucleosis and the diagnosis was further confirmed at the Oregon Public Health Laboratory by the presence of heterophile antibodies which were not removable by guinea pig kidney absorption. The titers are given in Table 6.

Twenty-five of the 61 total serum samples were normal human sera. They were obtained as specimens taken from healthy young women during pre-natal examinations or from healthy college students. Their total protein contents fell within the limits of 6.35-7.92 g/100 ml. Table 4 presents the complete analytic data for these specimens.

Tables 4, 5, and 6 give the results of the complete analysis of these normal and mononucleosis sera, in terms of percent composition and grams of protein/100 ml for each separated serum fraction. Results for the mononucleosis sera from Dr. MacHaffie and from Dr. Brandon of the Public Health Laboratory were grouped on separate tables because the first group consisted of positively diagnosed cases of infectious mononucleosis while the second group represented cases diagnosed presumptively by the Paul-Bunnell test and guinea pig kidney absorption.

To present a condensation of the data for comparison, Table 7 was constructed. It shows the differences in the range, means and standard deviations for the total protein content, and grams of protein/100 ml in the albumin, alpha-1, alpha-2, beta and gamma globulin fractions of each group of sera.

In comparing the normal group of sera in Table 4 to the group of infectious mononucleosis sera from the Public Health Laboratory, in Table 5, the following observations were made:

1. Total protein:

   Ten of the 25 samples of infectious mononucleosis serum have values above the upper limit of the range for normals (6.35-7.92 g/100 ml) but none of them are below the normal range. The mean value is above the mean for normals.

2. Albumin:

Four of the 25 mononucleosis sera are above the range of our normal sera (4.32-5.00 g/100 ml), while ten of them are below. The mean is very slightly below that for normals.

3. Alpha-1 globulin:

Most of the values are within the range of our normal sera (0.101-0.415 g/100 ml) except three samples, Nos. 5, 22, and 25 of which only No. 25 is significantly higher, because the value of the least significant difference determined for alpha-1 is 0.074 g/100 ml. The mean is above that for normals.

4. Alpha-2 globulin:

Nine of 25 infectious mononucleosis sera are above the normal range (0.489-0.893 g/100 ml). These are Nos. 1, 3, 5, 8, 12, 13, 14, 15, 20. However, only seven of these nine (Nos. 1, 8, 12, 13, 14, 15 and 20) are significantly above the normal range. None are below. The mean value is above the mean for normals.

5. Beta globulin:

Only one of the 25 mononucleosis sera is significantly above the normal range (0.429-0.890 g/100 ml). This is No. 12. None are below. The mean is slightly above that for normals.

6. Gamma globulin:

Twenty-one of the 25 are above the normal range (0. 757-1. 152 g/100 ml) but none are below. Eighteen of these 21 are significantly above because the least significant difference for gamma globulin has been determined to be 0. 097 g/100 ml. The mean is considerably higher than the value for normals.

Table 6 presents the analytical data for the 11 mononucleosis sera from the Student Health Service. Comparisons between this group and the normal sera in Table 4 give the following:

1. Total protein:

Five of the 11 mononucleosis sera have values above the upper limit of our normal range (6. 35-7. 92 g/100 ml), while none are below. The mean is also above the normal.

2. Albumin:

Two of the 11 mononucleosis sera are above our normal range (4. 32-5. 00 g/100 ml). These are Nos. 28 and 34. Two are below the normal range. These are Nos. 30 and 36. The mean is the same as that for normals.

3. Alpha-1 globulin:

All values are within the limit of our normal range (0. 101-0. 415 g/100 ml) except No. 36 which is above the upper limit but not high enough to be of significance.

The mean is slightly above normal.

4. Alpha-2 globulin:

Only two of the 11 mononucleosis sera are significantly above the upper limit of the normal range (0.489-0.893 g/100 ml). They are Nos. 29 and 31. None are below. The mean is slightly above normal.

5. Beta globulin:

None of the 11 specimens differ significantly from the normal range (0.429-0.890 g/100 ml). The mean is slightly above normal.

6. Gamma globulin:

Eight of the 11 mononucleosis sera (Nos. 26 through 33) have values significantly higher than the upper limit of our normal range (0.757-1.152 g/100 ml). None are below. The mean is well above normal.

Besides the quantitative differences outlined above, it was interesting to note that, even though the protein content of both the gamma and alpha-2 globulins was significantly increased in many mononucleosis samples, there seems to be very little correlation between this increase and the heterophile antibody titers of the sera. The reason for this is not clear.

Both groups of mononucleosis sera gave essentially similar results. A definite shift toward an increase in total serum proteins

was shown by the increase in the mean values compared to that of the normal sera, and the fact that in both groups slightly less than half of the specimens had a total protein content above the upper limit of the normals.

The data on individual fractions show that the increase is due to changes in the serum globulins and that the albumin content of the serum is unchanged.

The greatest increase in any single fraction is that of the gamma globulin, where about three-fourths of the values were above the upper limit of normals. The other globulin fractions are all increased slightly and to a similar extent when mean values are compared. However, only in the case of the alpha-2 globulin were some specimens found significantly above the normal range.

Table 4. Electrophoretic analysis of normal human sera.

Serum No.	albumin		α_1 globulin		α_2 globulin		β globulin		γ globulin		Total protein	Heterophile antibody
	%[1]	g/100 ml[2]	%	g/100 ml	%	g/100 ml	%	g/100 ml	%	g/100 ml	g/100 ml	titer
1	62.05	4.490	4.04	0.290	8.99	0.650	11.39	0.820	13.50	0.970	7.23	1:5
2	64.28	4.860	3.21	0.240	9.83	0.740	9.24	0.700	13.40	1.010	7.56	zero
3	62.00	4.680	4.39	0.330	8.81	0.670	11.33	0.850	13.44	1.020	7.56	zero
4	60.27	4.590	4.64	0.350	11.57	0.880	11.62	0.890	11.85	0.900	7.61	zero
5	68.28	4.548	1.81	0.121	10.07	0.671	7.21	0.481	12.62	0.841	6.66	zero
6	65.33	4.692	3.03	0.218	8.04	0.578	10.16	0.730	13.42	0.964	7.18	1:5
7	63.73	4.733	3.50	0.261	11.94	0.887	7.20	0.535	13.71	1.019	7.43	zero
8	65.14	4.960	3.23	0.246	8.50	0.647	8.46	0.645	14.64	1.115	7.61	zero
9	67.17	4.823	3.12	0.225	8.46	0.613	7.53	0.541	13.71	0.985	7.18	zero
10	64.40	4.692	4.59	0.335	12.25	0.893	8.34	0.608	10.38	0.757	7.26	zero
11	64.04	4.361	4.79	0.326	9.89	0.673	9.06	0.618	12.21	0.839	6.81	zero
12	69.02	4.728	2.88	0.197	7.16	0.489	8.41	0.576	12.54	0.859	6.85	zero
13	66.28	4.315	3.55	0.232	9.11	0.595	8.38	0.546	12.67	0.825	6.51	zero
14	64.48	4.589	4.52	0.321	8.11	0.577	9.68	0.689	13.20	0.939	7.12	zero
15	64.61	4.895	2.52	0.192	10.54	0.803	9.85	0.751	12.44	0.951	7.61	zero
16	68.54	4.769	1.93	0.135	9.83	0.684	6.72	0.466	12.99	0.904	6.96	1:5
17	65.12	4.337	2.36	0.182	9.35	0.623	8.67	0.577	14.72	0.980	6.66	zero
18	67.72	4.819	2.38	0.170	8.59	0.612	8.47	0.639	12.29	0.875	7.12	zero
19	71.44	4.532	1.59	0.101	7.98	0.505	6.77	0.429	12.16	0.772	6.35	zero
20	66.52	4.803	2.55	0.184	7.04	0.508	9.63	0.695	14.26	1.030	7.22	zero
21	63.11	4.998	5.24	0.415	10.45	0.827	9.29	0.736	11.87	0.941	7.92	zero
22	62.92	4.832	3.82	0.294	6.66	0.512	11.35	0.867	15.00	1.152	7.68	zero
23	60.55	4.372	5.21	0.376	11.31	0.817	8.57	0.619	14.32	1.034	7.22	zero
24	63.42	4.769	4.48	0.337	11.45	0.861	8.23	0.619	12.39	0.931	7.52	zero
25	62.10	4.714	5.29	0.402	11.41	0.866	8.29	0.629	12.88	0.977	7.59	zero
	60.27	4.315	1.59	0.101	6.66	0.489	6.72	0.429	10.38	0.757	6.35	
Range	71.44	4.998	5.29	0.415	12.25	0.893	11.62	0.890	15.00	1.152	7.92	
Mean	64.84	4.676	3.547	0.259	9.494	0.687	8.954	0.650	13.072	0.944	7.22	
St. dev.	2.731	0.193	1.125	0.086	1.632	0.131	1.401	0.112	1.056	0.095	0.413	

[1] Means percent of total serum protein.

[2] Means gram protein/100 ml of serum.

Table 5. Electrophoretic analysis of mononucleosis sera - supplied by Oregon Public Health Laboratory

Serum No.	albumin		α_1 globulin		α_2 globulin		β globulin		γ globulin		Total protein	Hetero-phile antibody titer[2]
	%[1]	g/100 ml	%	g/100 ml	%	g/100 ml	%	g/100 ml	%	g/100 ml	g/100 ml	
1	50.74	3.851	4.23	0.323	14.73	1.118	8.89	0.675	21.39	1.624	7.59	1:1280
2	60.94	5.081	3.68	0.307	8.35	0.697	10.11	0.843	16.90	1.409	8.34	1:100
3	55.20	4.244	3.54	0.272	11.71	0.901	9.30	0.715	20.21	1.558	7.69	1:320
4	60.81	4.171	5.46	0.374	9.54	0.657	10.16	0.697	14.00	0.961	6.86	1:1280
5	55.74	4.146	6.03	0.449	12.07	0.898	9.68	0.720	16.49	1.227	7.44	1:1280
6	58.76	4.277	4.61	0.336	11.72	0.853	10.17	0.740	14.74	1.073	7.28	1:320
7	57.38	4.061	3.94	0.279	11.28	0.798	9.49	0.672	17.89	1.266	7.07	1:320
8	56.28	4.868	4.56	0.395	11.81	1.022	10.66	0.923	16.67	1.442	8.65	1:1280
9	58.68	4.037	6.03	0.415	12.02	0.827	9.89	0.681	13.36	0.920	6.88	1:100
10	60.90	4.031	4.79	0.317	10.87	0.720	10.00	0.663	13.42	0.889	6.62	1:2500
11	58.14	4.431	4.74	0.361	6.58	0.501	10.28	0.783	20.25	1.543	7.62	1:100
12	52.00	4.550	2.89	0.253	11.27	0.986	12.17	1.065	21.66	1.896	8.75	1:2500
13	56.23	4.718	4.28	0.360	12.10	1.015	9.04	0.759	18.32	1.538	8.39	1:100
14	54.49	5.398	4.15	0.412	10.10	1.001	9.27	0.919	21.97	2.177	9.91	1:1280
15	66.24	6.624	3.50	0.351	10.21	1.021	7.67	0.767	12.37	1.237	10.00	1:640
16	53.72	4.443	4.22	0.349	10.39	0.860	8.35	0.691	23.30	1.927	8.27	1:2500
17	60.23	4.993	3.57	0.297	10.72	0.889	9.71	0.806	15.74	1.305	8.29	1:100
18	59.03	5.019	4.70	0.400	9.35	0.796	9.07	0.771	17.83	1.516	8.50	1:100
19	57.70	4.569	3.67	0.291	9.85	0.780	7.94	0.629	20.82	1.649	7.92	1:1280
20	56.28	4.424	5.03	0.396	9.99	0.785	12.10	0.951	16.58	1.304	7.86	1:640
21	60.24	4.615	5.26	0.404	13.70	1.050	7.95	0.609	12.83	0.983	7.66	1:1280
22	56.44	4.440	5.61	0.441	10.88	0.824	8.89	0.698	18.68	1.470	7.87	1:5120
23	56.54	4.088	4.28	0.347	10.46	0.760	10.75	0.781	17.46	1.262	7.27	1:2500
24	60.84	4.977	3.81	0.312	8.88	0.726	7.73	0.632	18.74	1.534	8.18	1:100
25	48.83	3.688	6.77	0.511	11.76	0.888	10.58	0.799	22.06	1.666	7.55	1:320
	48.83	3.688	2.89	0.253	6.58	0.501	7.67	0.609	12.37	0.889	6.62	
Range	66.24	6.624	6.77	0.511	14.73	1.118	12.17	1.065	22.06	2.177	10.00	
Mean	57.295	4.550	4.530	0.358	10.813	0.855	9.594	0.760	17.150	1.415	7.93	
St. dev.	3.161	0.629	0.934	0.062	1.667	0.148	1.076	0.112	3.163	0.326	0.84	

[1] Means percent of total serum protein and grams protein/100 ml of serum.
The least significant difference determined for analyses of alpha-1 globulin = 0.074 g/100 ml.
gamma globulin = 0.097 g/100 ml.

[2] These titrations were done by the Public Health Laboratory in Portland, given as values before absorption.

Table 6. Electrophoretic analysis of mononucleosis sera from the Student Health Service.

Serum No.	albumin		α_1 globulin		α_2 globulin		β globulin		γ globulin		Total protein g/100 ml	Hetero-phile titer[1]
	%[2]	g/100 ml	%	g/100 ml	%	g/100 ml	%	g/100 ml	%	g/100 ml		
26	57.70	4.953	3.83	0.330	9.63	0.830	10.81	0.931	18.19	1.567	8.61	1:2560
27	53.76	4.406	4.93	0.404	10.11	0.829	10.53	0.863	20.63	1.691	8.20	1:5120
28	60.94	5.081	3.68	0.307	8.35	0.697	10.11	0.843	16.90	1.409	8.34	1:640
29	56.24	4.718	4.28	0.360	12.10	1.015	9.04	0.759	18.32	1.538	8.39	1:320
30	59.94	4.256	4.22	0.299	8.56	0.608	8.67	0.616	18.56	1.318	7.10	1:1280
31	53.79	4.853	4.46	0.403	10.91	0.984	9.97	0.900	20.79	1.876	9.02	1:2560
32	58.29	4.463	3.14	0.240	8.04	0.615	10.44	0.799	20.07	1.535	7.65	1:640
33	59.52	4.487	4.62	0.348	7.28	0.549	10.36	0.781	18.20	1.372	7.54	1:1280
34	69.21	5.253	3.36	0.255	6.65	0.505	6.61	0.483	14.42	1.093	7.59	1:640
35	59.70	4.693	3.88	0.305	11.88	0.934	9.02	0.709	15.50	1.219	7.86	1:1280
36	55.71	4.128	6.09	0.452	12.09	0.896	9.34	0.692	16.75	1.241	7.41	1:2560
	53.76	4.128	3.14	0.240	6.65	0.505	6.61	0.483	14.42	1.093	7.10	
Range	69.21	5.253	6.09	0.452	12.10	1.015	10.81	0.931	20.79	1.876	9.02	
Means	58.610	4.663	4.226	0.337	9.600	0.769	9.530	0.762	18.030	1.442	7.97	
St. dev.	2.676	0.348	0.811	0.065	1.958	0.182	1.203	0.132	2.032	0.227	0.58	

[1] These titrations were done in our own laboratory. Titers are those of unabsorbed sera.

[2] Means percent of total serum protein and gram protein/100 ml of serum.
The least significant difference determined for analyses of: alpha-1 globulin = 0.074 g/100 ml.
gamma globulin = 0.097 g/100 ml.

Table 7. A direct comparison of the condensed analytical data for normal and mononucleosis sera in grams of protein of each serum fraction per 100 ml.

	albumin	α_1 globulin	α_2 globulin	β globulin	γ globulin	Total protein
25 Normal Sera						
Range	4.315-4.998	0.101-0.415	0.489-0.893	0.429-0.890	0.757-1.152	6.35-7.92
Means	4.676	0.259	0.687	0.650	0.944	7.22
St. deviation	0.193	0.086	0.131	0.112	0.095	0.413
25 Mono. Sera						
Range	3.688-6.624	0.253-0.511	0.501-1.118	0.609-1.065	0.889-2.177	6.62-10.00
Means	4.550	0.358	0.855	0.760	1.415	7.93
St. deviation	0.629	0.062	0.148	0.112	0.326	0.84
11 Mono. Sera						
Range	4.128-5.253	0.240-0.452	0.505-1.015	0.483-0.931	1.093-1.876	7.10-9.02
Means	4.663	0.337	0.769	0.762	1.442	7.97
St. deviation	0.348	0.065	0.182	0.132	0.227	0.58

The Localization of the Heterophile Antibody in Electrophoretic Fractions of the Mononucleosis Sera

Franger (16), in his work with infectious mononucleosis sera, was able to demonstrate the presence of heterophile antibodies in the gamma globulin fraction by using a hemagglutination technique carried out directly on paper strips which bore the electrophoretically separated fractions. We have modified this method by using cellulose acetate strips, with the same purpose in mind, to localize the exact fraction or fractions which contain these antibodies.

Twenty-two mononucleosis sera were studied in this way. These sera were obtained from the Oregon Public Health Laboratory in Portland with their heterophile antibody titers already determined. They were first subjected to electrophoretic analysis on cellulose acetate strips. Each separated fraction was eluted from the strip as described previously with physiological saline and tested undiluted for ability to agglutinate sheep erythrocytes. The electrophoretic analysis of each is presented in Table 8.

It will be noted that in 21 out of the 22 cases, the eluted gamma globulin fraction agglutinated the sheep cells. Hemagglutination also occurred with the alpha-2 globulin fraction in ten of the serum specimens. In nine of these ten specimens, the alpha-2 globulin content was above the upper limit of the normal range as found in Table 8, i. e., 0. 89 g/100 ml. Furthermore, all specimens that gave a

definitely negative reaction had an alpha-2 globulin value within the range of normal values. However, in two cases where the globulin content appeared above normal, no definite hemagglutination was observed (i. e., serum No. 13 and No. 20).

It is interesting to note, through careful examination of the data, that the presence of the hemagglutinins in the alpha-2 fraction of the globulin bears no relationship to the heterophile titer; e. g., in serum Nos. 4 and 10, where the titer was 1:640, there was hemagglutinin in the alpha-2 fraction. In Nos. 6, 8, 17, and 21, in which no hemagglutination occurred, the heterophile titer was also 1:640. No. 22 has a lower titer of 1:320 but pronounced hemagglutination took place.

These results show very clearly that the heterophile antibody of infectious mononucleosis was found almost always in the gamma globulin and in almost half the cases was also present in the alpha-2 globulin fraction. These results differ slightly from those of Frenger (16), who found the antibody only in the gamma globulin. This difference could be due to a possibly greater sensitivity of the method of testing for hemagglutinin employed in this study.

Table 8. Localization of the heterophile antibody in mononucleosis sera with respect to electrophoretic fractions.

		Electrophoretic analysis (g/100 ml)					Hemagglutination of sheep erythrocytes after elution of fraction from strip				
Serum No.	Heterophile titer[1]	Albumin	α_1 globulin	α_2 globulin	β globulin	γ globulin	Albumin	α_1 globulin	α_2 globulin	β globulin	γ globulin
1	1:5120	4.798	0.345	0.926	0.695	1.607	-[2]	-	+	-	++
2	1:2560	4.853	0.403	0.984	0.900	1.876	-	-	+	-	++
3	1:160	4.405	0.289	0.816	0.509	1.199	-	-	-	-	++
4	1:640	4.466	0.419	0.897	0.700	1.782	-	-	+	-	++
5	1:2560	4.931	0.314	0.997	0.797	1.667	-	-	++	-	++
6	1:640	4.256	0.299	0.608	0.616	1.318	-	-	-	-	++
7	1:2560	4.910	0.335	0.898	0.898	1.652	-	-	±	-	++
8	1:640	4.433	0.346	0.923	1.096	1.777	-	-	+	-	++
9	1:10240	4.745	0.526	0.969	0.647	1.312	-	-	±	-	++
10	1:640	4.451	0.465	0.978	0.769	1.841	-	-	+	-	++
11	1:2560	5.466	0.357	0.695	0.838	1.573	-	-	-	-	++
12	1:10240	4.274	0.265	1.328	0.739	1.733	-	-	++	-	++
13	1:1280	5.363	0.423	1.016	0.621	0.988	-	-	-	-	-
14	1:5120	4.369	0.371	0.960	0.650	1.285	-	-	+	-	++
15	1:5120	3.999	0.309	0.739	0.826	1.029	-	-	-	-	++
16	1:2560	4.606	0.431	1.097	0.454	1.138	-	-	+	-	++
17	1:640	4.329	0.348	0.876	0.726	1.991	-	-	-	-	++
18	1:320	3.688	0.510	0.888	0.799	1.666	-	-	-	-	++
19	1:160	4.977	0.312	0.726	0.633	1.533	-	-	-	-	++
20	1:5120	4.963	0.259	0.910	0.992	1.489	-	-	±	-	++
21	1:640	4.081	0.307	0.697	0.843	1.409	-	-	-	-	++
22	1:320	4.718	0.300	1.015	0.759	1.538	-	-	++	-	++

[1] These titers are values before absorption with guinea pig kidney.

[2] - indicates no agglutination.

++ indicates complete agglutination.

+ indicates less agglutination as compared to ++.

± indicates doubtful agglutination, borderline cases.

The Effect of Absorption with Beef Erythrocytes on the Electrophoretic Pattern of Mononucleosis Sera

Heterophile antibodies of the infectious mononucleosis type are absorbed by beef erythrocytes (47, 13, 31, 30). Our electrophoretic analysis of the 36 mononucleosis sera, when compared to the 25 normal sera, indicates a very significant elevation in the protein content of the gamma globulin fraction, and a less pronounced, but also significant increase in the alpha-2 globulin fraction. In order to relate these elevations in protein to the presence of the heterophile antibodies, particularly the sheep cell agglutinins, we have conducted eight absorption experiments on eight different mononucleosis sera with heterophile titers ranging from 1:320 to 1:5120, using fresh raw beef erythrocytes.

These eight mononucleosis specimens, obtained from the Oregon Public Health Laboratory, with their heterophile titers already determined, were first analyzed electrophoretically to establish the protein content of each fraction in g/100 ml. The sera were then subjected to absorption with beef erythrocytes at 4^{o} C overnight, as described in detail in the Materials and Methods section. The absorbed specimens were dried, reconstituted to their original total solids content and examined by the Paul-Bunnell heterophile antibody titration; they were then subjected to another electrophoretic separation to compare the patterns with those before absorption.

Table 9 gives an overall picture of the electrophoretic patterns of each specimen before and after absorption.

This table shows a 100% absorption of the sheep red cell agglutinins in 50% of the samples, i. e., four samples out of eight. The other four retained a titer of 1:5.

In each case there was a significant reduction in the protein content of the gamma globulin fraction after absorption. The alpha-2 globulin was also reduced in all eight sera but the difference can be considered significant in only four of the eight, i. e., in 50% of the cases (Nos. 1, 2, 5, and 8). The other fractions were either increased slightly or unchanged after absorption. This would be expected from the reduction of the alpha-2 and gamma globulins because the absorbed and dried sera were reconstituted to the original concentration of total solids.

These findings confirm the results of the direct experiment on localization of the heterophile antibodies, or to be specific, the sheep cell agglutinins of infectious mononucleosis sera, indicating by a different experimental approach that they are mainly in the alpha-2 and gamma globulin fractions of the serum protein. These antibodies can be completely absorbed, or nearly so, by beef erythrocytes. The gamma globulin, and in some sera, the alpha-2 globulin were the only two fractions whose protein content was consistently and significantly reduced by the absorption.

Table 9. Effect of absorption with beef erythrocytes on the electrophoretic pattern of mononucleosis sera.

	Electrophoretic analysis before absorption [1]							Electrophoretic analysis after absorption						
Serum No.	Hetero-phile titers	albumin	α_1 globulin	α_2 globulin	β globulin	γ globulin	Total protein g/100 ml	Hetero-phile titers	albumin	α_1 globulin	α_2 globulin	β globulin	γ globulin	Total protein g/100 ml [2]
1	1:2560	4.931	0.314	0.997	0.797	1.667	8.61	1:5	5.526	0.326	0.871	0.803	1.087	8.61
2	1:640	4.466	0.419	0.897	0.700	1.782	8.27	1:5	5.007	0.394	0.821	0.928	1.042	8.27
3	1.160	4.405	0.289	0.816	0.509	1.199	7.22	0	4.588	0.272	0.763	0.527	0.934	7.22
4	1:5120	4.798	0.345	0.926	0.695	1.607	8.38	1:5	5.155	0.299	0.870	0.779	1.225	8.33
5	1:2560	4.953	0.330	0.830	0.931	1.567	8.61	0	5.373	0.423	0.699	0.987	1.131	8.61
6	1:2560	4.406	0.404	0.829	0.863	1.691	8.61	1:5	4.972	0.371	0.791	0.935	1.125	8.22
7	1:640	5.081	0.307	0.697	0.843	1.409	8.34	0	5.147	0.459	0.672	0.932	1.105	8.34
8	1:320	4.718	0.360	1.015	0.759	1.538	8.39	0	4.842	0.537	0.850	0.999	1.130	8.39

[1] All values given are mean values of four replicate strips.

[2] Analyzed by Biuret total protein determination.

The least significant difference between analyses of alpha-1 globulin is 0.074 g/100 ml.
gamma globulin is 0.097 g/100 ml.

Study of Mononucleosis Sera by Immunoelectrophoresis

Immunoelectrophoresis offers much better resolution in serum protein separation than regular electrophoresis. By using this method, we aimed to obtain more information about the constituents of each serum fraction in a mononucleosis specimen compared to that of a normal specimen.

This procedure consists of two main parts. The first part is a regular electrophoretic separation of the serum specimen in a buffered agar gel. The separated pattern is then subjected to a second stage of analysis in which specific antiserum against human serum is added to a trough in the agar parallel to the separated pattern to allow precipitin reactions to take place between the separated antigens and their antibodies.

Twelve mononucleosis sera from the Oregon Public Health Laboratory and 12 normal sera were used for this purpose. The electrophoretic analysis of these sera on cellulose acetate is presented in Table 10. The results of immunoelectrophoresis are outlined in Table 11, and in the photographs of Figures 3 to 8. Each specimen was analyzed using antibody prepared in rabbits against normal human serum and also antibody against mononucleosis serum.

Two very significant findings are apparent in the patterns obtained on these 12 pairs of immunoelectrophoretic plates:

1. The alpha-2 globulins:

In 11 of the 12 mononucleosis specimens used, an abnormal pattern was observed in the alpha-2 globulin zone. Regardless of the type of antibody used for analyzing the mononucleosis sera, i. e. antibody against normal human serum or antibody against mononucleosis serum, the number of fractions resolved varied from three to six in different specimens. The same variation was observed in the 11 normal sera which were analyzed at the same time.

The abnormal pattern, however, in all the mononucleosis specimens was characterized by the fact that one or more of the alpha-2 globulin arcs were longer than any of those from the normal specimens. These extended well into the beta-1 globulin zone. When analysis was made using antibody against mononucleosis serum, the arc or arcs were usually the ones most distant from or in the second most distant position from the central antibody trough (see Figures 3 and 4, and Table 11). When analysis was made using antibody against normal serum, more than one extended arc was usually observed. Their positions varied from the nearest to the most distant from the antibody trough (see Figures 5 and 6, and Table 11). These extended arcs in the alpha-2 fraction were not observed in 11 of the 12 normal specimens studied. In one case, however, a single extended arc was noted when the antibody against mononucleosis serum was used, but not with antibody against normal serum.

These observations seem to indicate that in the mononucleosis sera analyzed, one or sometimes two of the alpha globulin components had been altered to include a population of molecules with a wider than normal range of electrophoretic mobilities, resulting in extension of the corresponding precipitin arcs into the beta-1 globulin zone. The altered components cannot represent antigenically new globulins because they react with antibody against normal human serum as well as that prepared against mononucleosis serum. It seems probable that these altered alpha-2 globulins may contain heterophile antibody, as such antibody was found in the alpha-2 fraction in ten of 22 mononucleosis cases, as previously described in our experiments using hemagglutination by the eluates of fractions from cellulose acetate strips.

2. The Beta-2M globulin:

Another very significant difference in the mononucleosis specimens when they were analyzed against anti-normal human serum and anti-mononucleosis serum was the fact that the precipitin arcs representing the beta-2M globulin in these plates were more heavily stained and more distinct when compared to the 12 normal sera. This occurred in ten cases where antibody against normal human serum was used, and in five cases where antibody against mononucleosis serum was used (see Figures 7 and 8, and Table 11). The beta-2M globulin fraction is recognized as an antibody containing fraction.

Our results on cellulose acetate analysis have shown a significant increase of the gamma globulin in 29 of 36 cases of mononucleosis studied. This fraction on cellulose acetate very probably contains most of the beta-2M globulin. Thus, although the immunoelectrophoretic analyses are not quantitative, the more heavily stained beta-2M globulin arcs may well be associated with the greater gamma globulin concentration on cellulose acetate. According to Orjan Strannegard and Erick Lycke (45) the mononucleosis antibodies precipitating the ox cell antigen were in the beta-2M globulin fraction. This observation was made by using agar diffusion immunoelectrophoresis.

A third observation from our immunoelectrophoretic analyses was the presence of a heavy pre-albumin arc that extended along the side of the albumin fraction. This does not seem to have any significance in our mononucleosis cases because both the mononucleosis and normal serum specimens showed this pattern when reacted with antibodies against mononucleosis serum, but not with antibody against normal serum.

Table 10. Electrophoretic analysis on cellulose acetate of mononucleosis sera[1/] used for agar gel immunoelectrophoresis.

Serum No.	albumin % [3/]	albumin g/100 ml	α_1 globulin %	α_1 globulin g/100 ml	α_2 globulin %	α_2 globulin g/100 ml	β globulin %	β globulin g/100 ml	γ globulin %	γ globulin g/100 ml	Total protein g/100 ml	Heterophile antibody titer [2/]
5	59.70	4.692	3.52	0.277	11.51	0.905	9.28	0.729	15.99	1.257	7.86	1:1280
7	56.21	4.716	4.66	0.391	11.86	0.995	8.90	0.747	18.36	1.540	8.39	1:160
9	53.93	5.342	4.92	0.487	9.96	0.987	9.03	0.895	22.16	2.195	9.91	1:1280
13	59.90	4.996	4.69	0.389	10.21	0.846	9.79	0.812	15.42	1.278	8.29	1:100
15	55.73	4.38	6.32	0.497	10.80	0.849	11.07	0.870	16.07	1.263	7.86	1:640
16	55.49	4.394	4.19	0.332	10.84	0.859	7.80	0.618	21.68	1.717	7.92	1:1280
17	50.90	3.863	2.40	0.182	15.97	1.212	9.38	0.712	21.36	1.621	7.59	1:1280
18	54.92	4.223	4.84	0.372	11.35	0.873	9.68	0.744	19.21	1.477	7.69	1:320
19	60.76	4.168	4.65	0.319	10.67	0.732	9.81	0.673	14.10	0.968	6.86	1:1280
20	55.07	4.097	5.55	0.413	12.14	0.903	9.92	0.738	17.32	1.289	7.44	1:1280
21	60.16	5.017	3.58	0.298	9.43	0.786	9.43	0.786	17.40	1.450	8.34	1:640
22	59.11	4.52	3.44	0.263	8.00	0.658	12.01	0.919	18.83	1.440	7.65	1:640

[1/] These 12 serum specimens were obtained from the Public Health Laboratory, Portland, Ore.

[2/] These titrations were done by the Oregon Public Health Laboratory.

[3/] Means percent of total serum protein.

Table 11. Results of immunoelectrophoresis of mononucleosis and normal sera.

	Alpha-2 Globulin		Beta-2M Globulin	
	Antibody against normal serum	Antibody against mononucleosis serum	Antibody against normal serum	Antibody against mononucleosis serum
Mononucleosis serum No. 5	6 fractions;[1] No. 4 extends into beta-1 zone.	5 fractions; No. 2 extends into beta-1 zone.	arc heavier and more distinct.	arc more distinct.
Normal serum No. 5N	4 fractions; in alpha-2 zone only.	5 fractions in alpha-2 zone only.	faint arc.	diffuse arc.
Mononucleosis serum No. 9	3 fractions; No. 3 extends into beta-1 zone.	5 fractions; No. 2 extends into beta-1 zone.	-------	arc heavier and more distinct.
Normal serum No. 9N	4 fractions; in alpha-2 zone only.	5 fractions in alpha-2 zone only.	-------	arc faint and diffuse.
Mononucleosis serum No. 13	6 fractions; No. 4 extends into beta-1 zone.	5 fractions; No. 2 extends into beta-1 zone.	arc heavier and more distinct.	arc definite.
Normal serum No. 13N	4 fractions; in alpha-2 zone only.	5 fractions in alpha-2 zone only.	faint arc.	definite arc.
Mononucleosis serum No. 7	4 fractions; No. 2 extends into beta-1 zone.	4 fractions; No. 2 extends into beta-1 zone.	arc heavier and more distinct.	arc more distinct.
Normal serum No. 7N	5 fractions all in alpha-2 zone only.	6 fractions in alpha-2 zone.	faint arc.	--------
Mononucleosis serum No. 15	4 fractions; No. 1 and 3 extend into beta-1 zone.	4 fractions; No. 2 and 3 extend into beta-1 zone.	arc heavier and more distinct.	arc definite.
Normal serum No. 15N	3 fractions in alpha-2 zone only.	4 fractions in alpha-2 zone only.	faint arc.	very faint arc.
Mononucleosis serum No. 16	5 fractions; No. 1 and 3 extend into beta-1 zone.	5 fractions; No. 1 extends into beta-1 zone.	arc heavier and more distinct.	diffuse arc.
Normal serum No. 16N	5 fractions in alpha-2 zone only.	5 fractions in alpha-2 zone only.	faint arc.	diffuse arc.

Table 11. Continued.

	Alpha-2 Globulin		Beta-2M Globulin	
	Antibody against normal serum	Antibody against mononucleosis serum	Antibody against normal serum	Antibody against mononucleosis serum
Mononucleosis serum No. 17	5 fractions; No. 4 extends into beta-1 zone.	5 fractions; No. 1 extends into beta-1 zone.	arc slightly heavier and more distinct.	±
Normal serum No. 17N	4 fractions in alpha-2 zone only.	4 fractions in alpha-2 zone only.	definite arc.	±
Mononucleosis serum No. 18	4 fractions; No. 2 and 4 extend into beta-1 zone.	5 fractions; No. 2 extends into beta-1 zone.	arc more distinct and heavier.	diffuse arc.
Normal serum No. 18N	4 fractions in alpha-2 zone only.	5 fractions in alpha-2 zone only.	faint arc.	diffuse arc.
Mononucleosis serum No. 19	4 fractions; No. 1 and 3 extend into beta-1 zone.	5 fractions; No. 2 extends into beta-1 zone.	arc heavier and more distinct.	arc more distinct.
Normal serum No. 19N	3 fractions in alpha-2 zone only.	5 fractions in alpha-2 zone only.	definite arc.	diffuse arc.
Mononucleosis serum No. 20	4 fractions, No. 1, 2 and 3 extend into beta-1 zone.	5 fractions; No. 1 extends into beta-1 zone.	arc heavier and more distinct.	±
Normal serum No. 20N	5 fractions in alpha-2 zone only.	6 fractions; No. 2 extends nearly into beta-1 zone.	definite arc.	±
Mononucleosis serum No. 21	4 fractions; No. 1 and 4 extend into beta-1 zone.	4 fractions; No. 4 extends into beta-1 zone.	definite arc.	diffuse arc.
Normal serum No. 21N	5 fractions in alpha-2 zone only.	5 fractions in alpha-2 zone only.	definite arc.	diffuse arc.
Mononucleosis serum No. 22	4 fractions; No. 2 extends into beta-1 zone.	4 fractions; No. 1 extends into beta-1 zone.	arc heavier and more distinct.	arc heavier and more distinct.
Normal serum No. 22 N	4 fractions in alpha-2 zone only.	6 fractions in alpha-2 zone only.	faint arc.	faint and diffuse arc.

1/ No. 1 indicates arc most distant from antibody trough; No. 2 is second; No. 3 third in position.

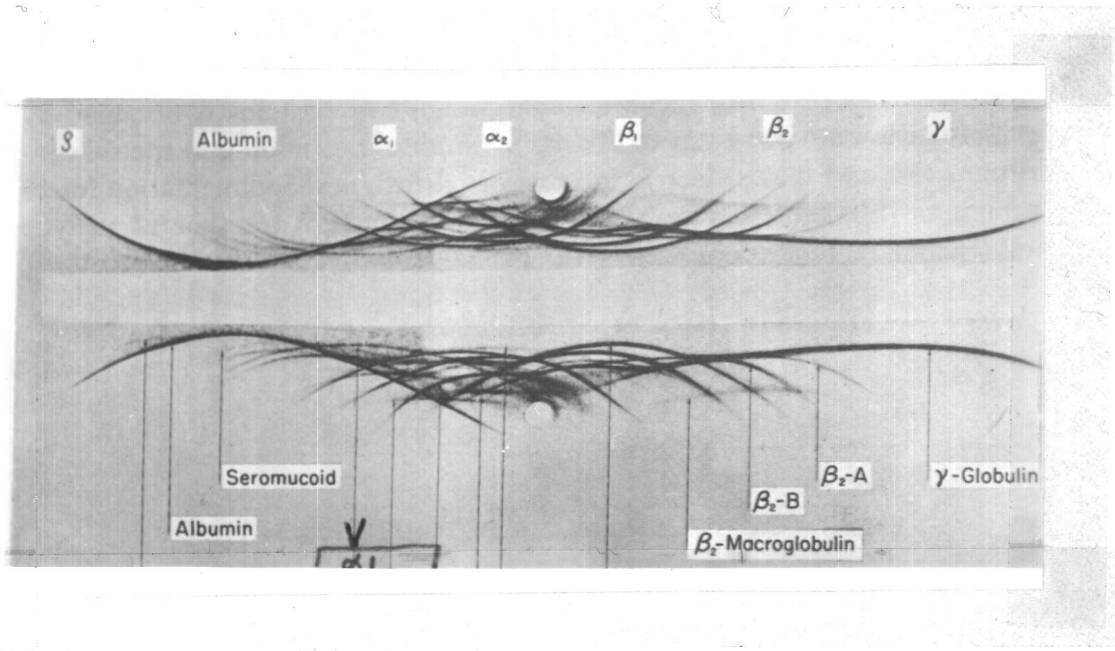

Figure 3. Diagram showing positions of precipitating arcs on immuno-electrophoretic plates.

Figure 3A. Mononucleosis serum No. 5 in upper half.
Normal serum No. 5N in lower half.
Antibody against normal serum in central trough.

Figure 3B. Normal serum No. 5N in upper half.
Mononucleosis serum No. 5 in lower half.
Antibody against mononucleosis serum in central trough.

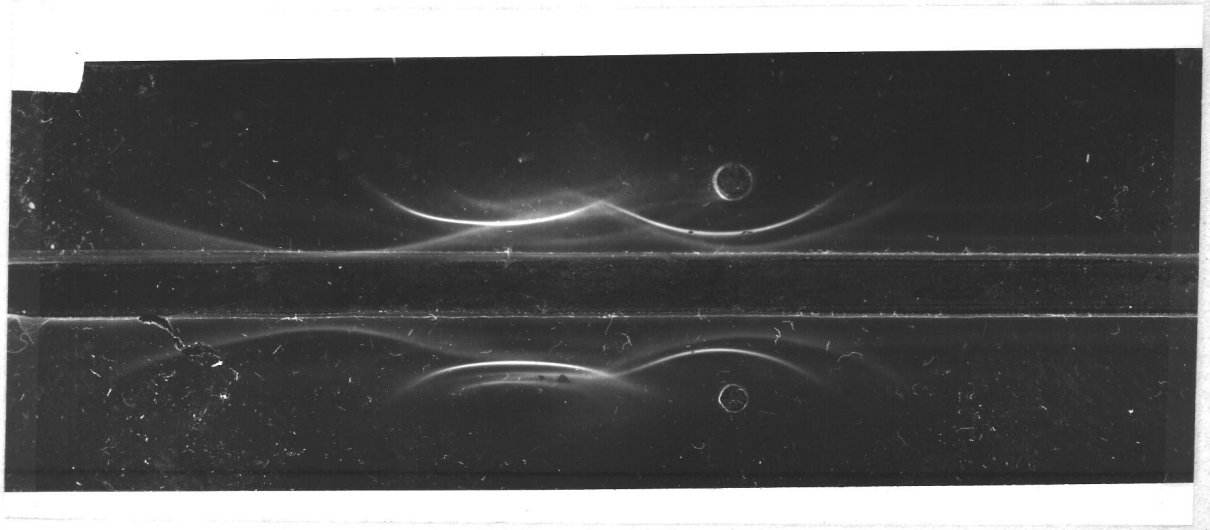

Figure 3A

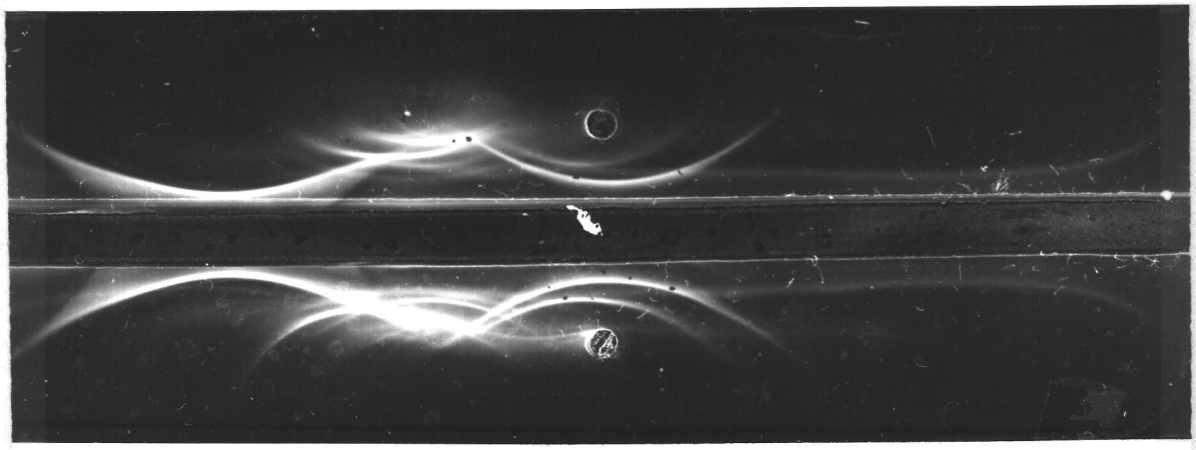

Figure 3B

Figure 4A. Mononucleosis serum No. 9 in upper half.
Normal serum No. 9N in lower half.
Antibody against normal serum in central trough.

Figure 4B. Normal serum No. 9N in upper half.
Mononucleosis serum No. 9 in lower half.
Antibody against mononucleosis serum in central trough.

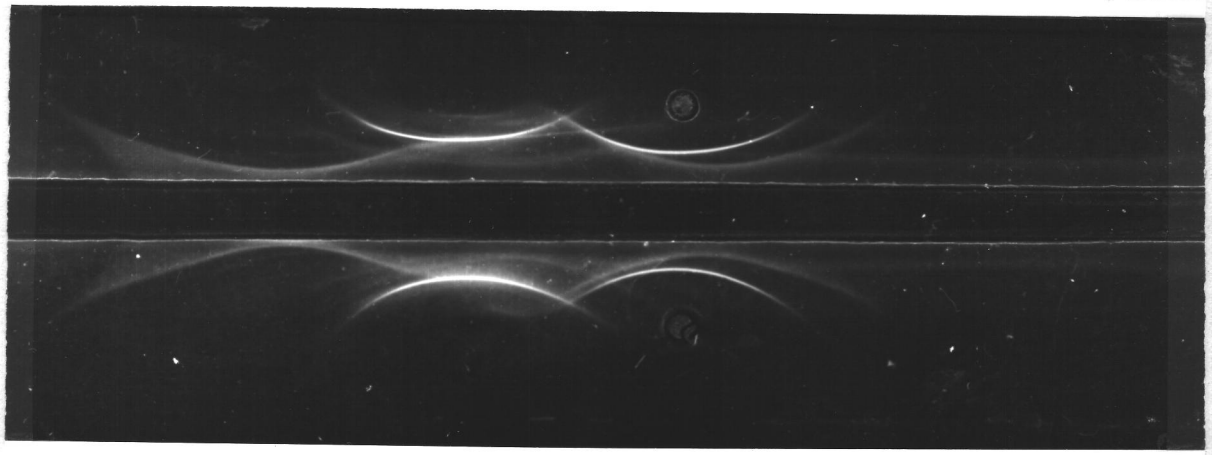

Figure 4A

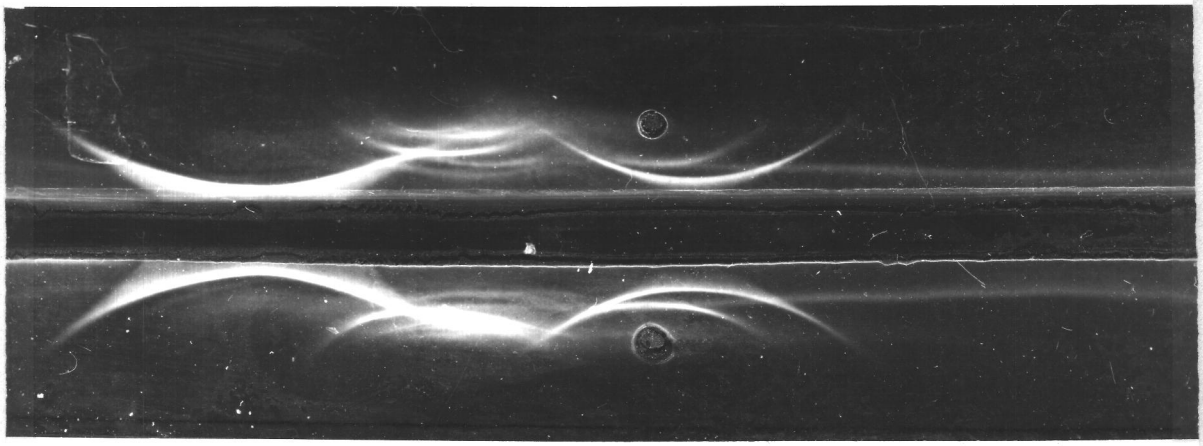

Figure 4B

Figure 5A. Mononucleosis serum No. 15 in upper half.
Normal serum No. 15N in lower half.
Antibody against normal serum in central trough.

Figure 5B. Normal serum No. 15N in upper half.
Mononucleosis serum No. 15 in lower half.
Antibody against mononucleosis serum in central trough.

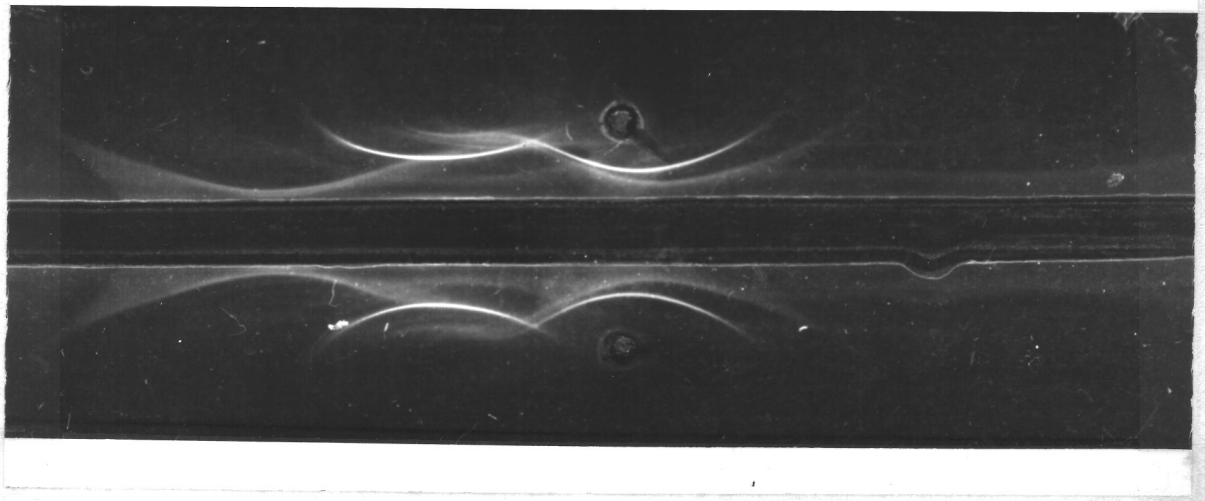

Figure 5A

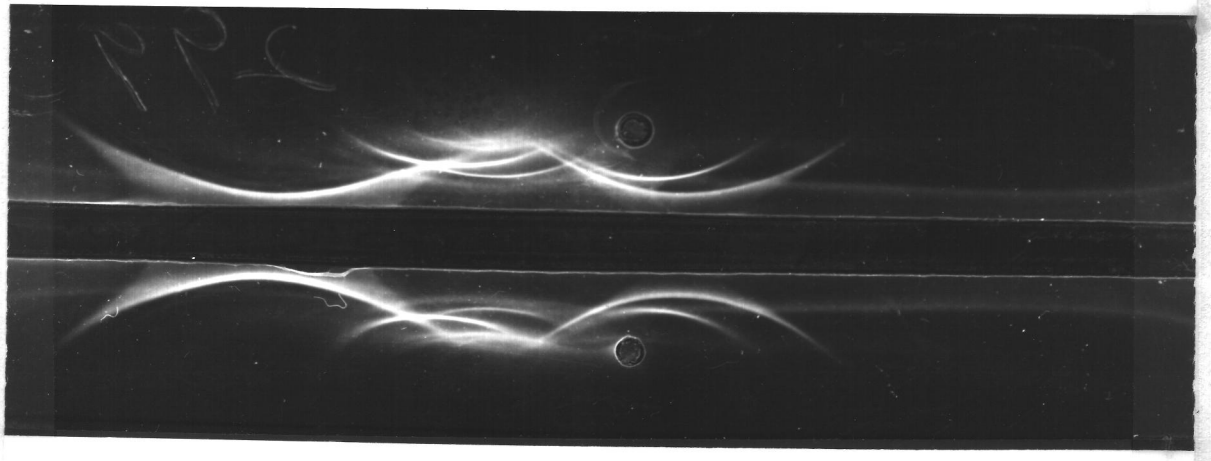

Figure 5B

Figure 6A. Mononucleosis serum No. 16 in upper half.
Normal serum No. 16N in lower half.
Antibody against normal serum in central trough.

Figure 6B. Normal serum No. 16N in upper half.
Mononucleosis serum No. 16 in lower half.
Antibody against mononucleosis serum in central trough.

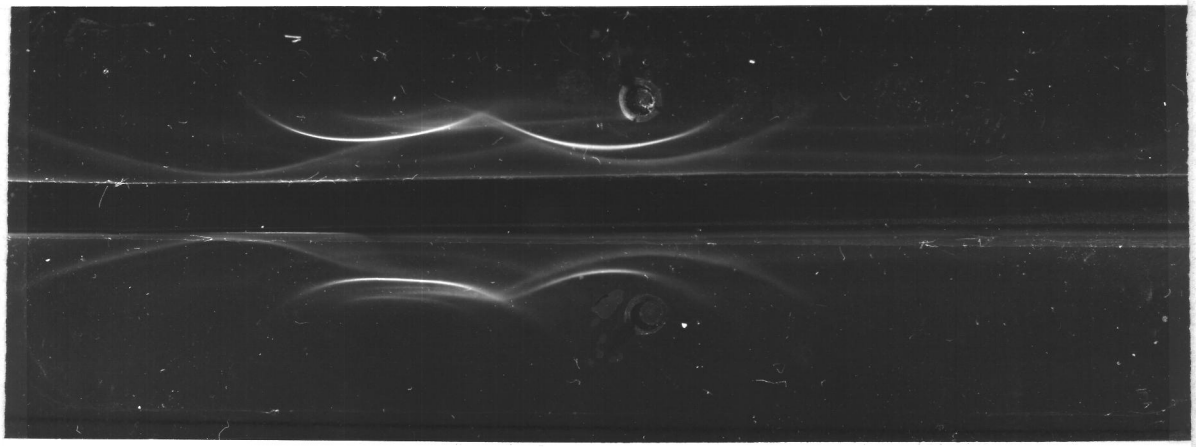

Figure 6A

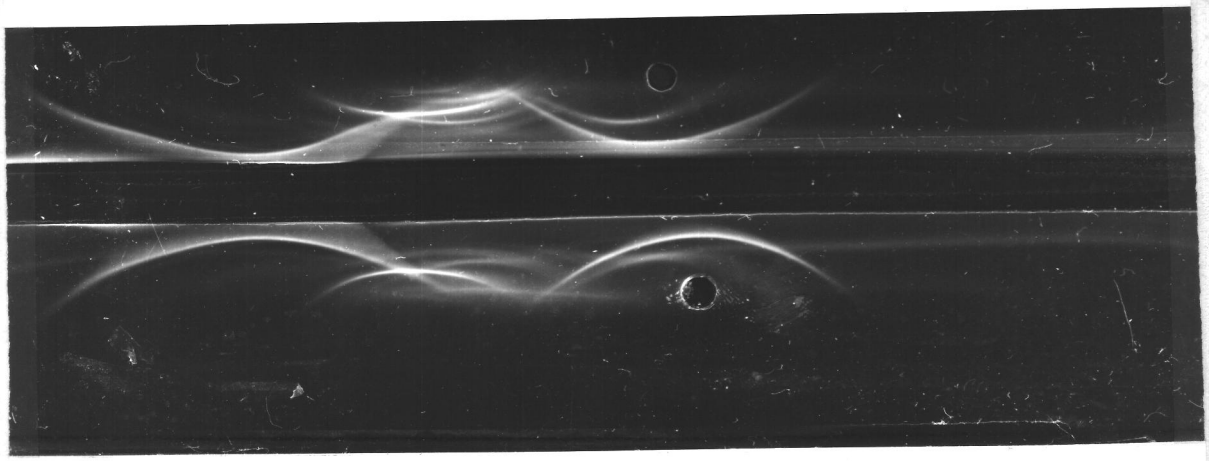

Figure 6B

Figure 7A. Mononucleosis serum No. 7 in upper half.
Normal serum No. 7N in lower half.
Antibody against normal serum in central trough.

Figure 7B. Normal serum No. 7N in upper half.
Mononucleosis serum No. 7 in lower half.
Antibody against mononucleosis serum in central trough.

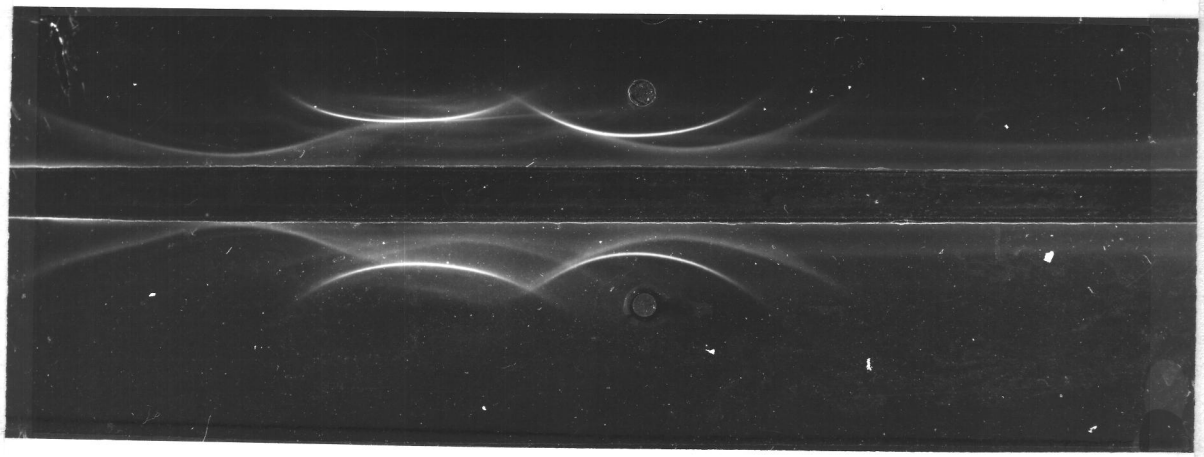

Figure 7A

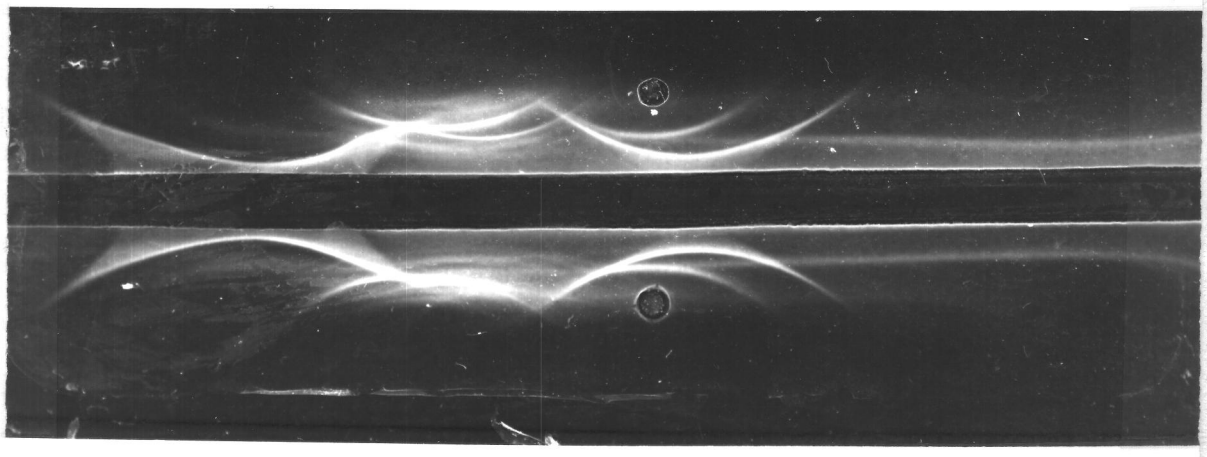

Figure 7B

Figure 8A. Mononucleosis serum No. 22 in upper half.
Normal serum No. 22N in lower half.
Antibody against normal serum in central trough.

Figure 8B. Normal serum No. 22N in upper half.
Mononucleosis serum No. 22 in lower half.
Antibody against mononucleosis serum in central trough.

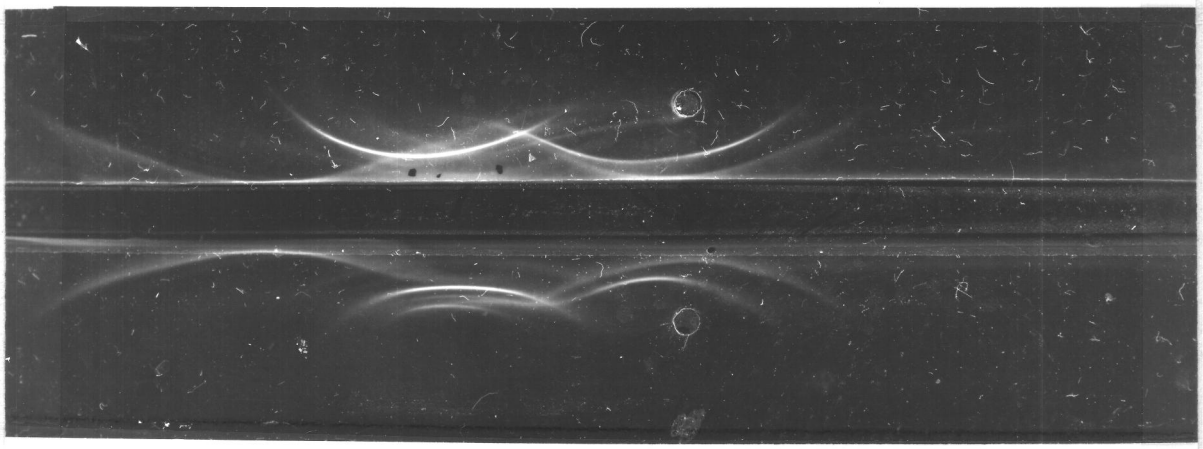

Figure 8A

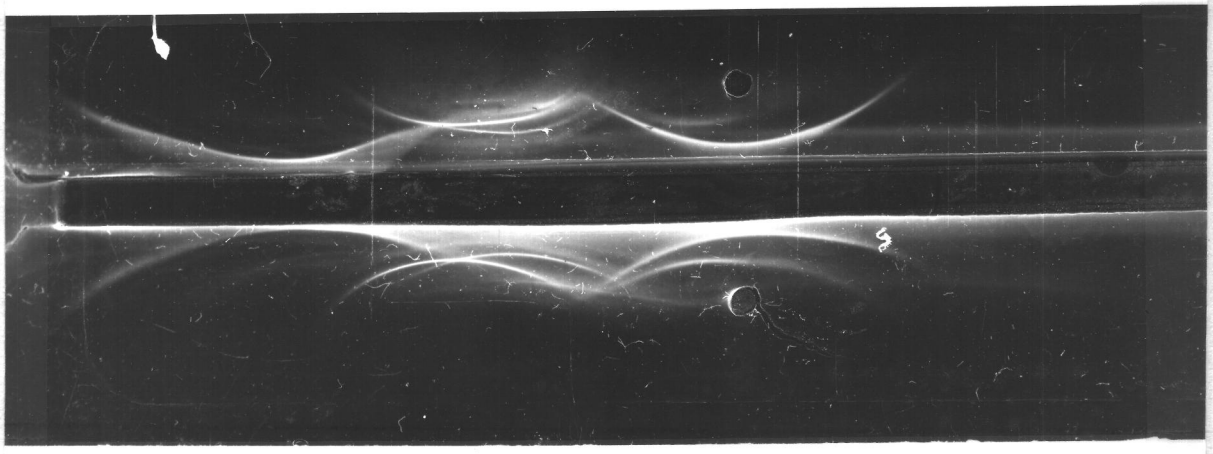

Figure 8B

DISCUSSION

The results of this investigation suggested that the heterophile antibodies, in terms of sheep cell agglutinins, occurring in sera from patients with infectious mononucleosis, were in any one or all of the following globulins:

1. The alpha-2 globulins:

The quantitative increase in the alpha-2 globulin fraction of infectious mononucleosis sera was removable by beef erythrocytes absorption. According to Layton (30) and Davidsohn (13), this is a characteristic of both the sheep cell agglutinins and the ox cell hemolysins. Our hemagglutination experiments also gave evidence for the existence of these sheep cell agglutinins in the alpha-2 region. Strannegard and Lycke (45) suggested that there is an antigenic factor common to both ox and sheep cells which will react with the infectious mononucleosis sera, and, there are two antigenic factors in ox erythrocytes. Therefore the Paul-Bunnell test is a test for more than one antibody. An Italian worker, Giuliani (19), in his analysis of 34 mononucleosis specimens, found 19 cases to be elevated in the alpha-2 globulin. No specific effort was made to credit this elevation to any kind of antibody. In our own immunoelectrophoretic experiments in agar gel, one or more alpha-2 arcs were found to extend into the beta-1 globulin zone. Further studies are required to demonstrate

whether these contain pure sheep cell agglutinin or a mixture.

2. The beta-2M globulins:

Besides our immunoelectrophoretic results which suggested a stronger beta-2M globulin in serum of infectious mononucleosis, Eyquem (15) has demonstrated that in 64 mononucleosis sera, 42 showed a significant increase in the beta-2M globulin while 12 showed a moderate increase. Franger (16), using hemagglutination directly on paper electrophoresis strips, found some agglutination in the beta globulin fraction, as well as the gamma globulin. Strannegard (45), by using agar gel electrophoresis, was able to demonstrate that the sheep cell agglutinins of infectious mononucleosis sera have an electrophoretic mobility similar to the human serum beta-2M globulin. Later in 1964, he was able to show the same mobility with the ox cell hemolysins. Pike (38), with DEAE cellulose chromatography, found that mononucleosis antibodies could not sensitize sheep cells for agglutination with the rheumatoid factor. The explanation for this should be that the infectious mononucleosis antibodies were 19 S gamma globulin while the rheumatoid factor was specific for 7 S gamma globulin and therefore gave no reaction with the cells coated with 19 S antibodies. The 19 S gamma globulin is identical with beta- 2M globulin.

3. The gamma globulins:

With less sensitive methods, like paper electrophoresis used by Franger (16), moving boundary electrophoresis by Sterling (44), another type of electrophoresis by Guiliani (19) and our own electrophoresis on cellulose acetate, the gamma globulin fraction seemed to be associated with the heterophile antibodies of infectious mononucleosis. Whether this serum fraction also contains the antibodies or the observation was actually due to the beta-2M globulin which is not distinguishable from the gamma globulin in the methods mentioned above is a phase of this problem that requires more intensive study.

SUMMARY AND CONCLUSIONS

I. Thirty-six sera from cases of infectious mononucleosis and 25 normal human serum specimens were studied by electrophoresis on cellulose acetate. The results indicated that:

A. The mean concentration of total protein in the mononucleosis sera was significantly higher than that of the normal sera. In 15 of the 36 mononucleosis specimens the total protein concentration was above the range of the normals.

B. The higher protein concentration was due entirely to increased amounts of the globulin fractions. The gamma globulin contributed most to the increase. Its mean concentration was above normal and in 29 of the 36 cases the gamma globulin level was above the range for the normal sera.

C. With respect to the alpha-2 globulin, the mean concentration was slightly above that for the normals and in nine of the 36 sera the concentration was above the normal range.

D. The mean concentration of the alpha-1 and beta globulins was slightly higher than the corresponding means for the normals.

II. Twenty-two mononucleosis sera were studied by electrophoresis on cellulose acetate followed by elution of each separate fraction and testing of the eluates for agglutination of sheep erythrocytes. The gamma globulin fraction of 21 of the 22 specimens agglutinated the

sheep cells. The alpha-2 globulin fraction of ten mononucleosis specimens also showed hemagglutination. Nine of these ten sera had an alpha-2 globulin content above the upper limit of normal (0. 89 g/100 ml). These results indicated that the heterophile antibodies were present in the gamma globulin fraction in almost all cases and in the alpha-2 globulin in about half the cases.

III. In experiments in which eight mononucleosis sera were absorbed with beef erythrocytes, complete removal of the heterophile antibodies was observed in four specimens while four others retained a low titer of 1:5. The significant reduction of the gamma globulin fraction in all eight cases and of the alpha-2 fraction in four cases after absorption, with no reduction in other fractions indicates that the heterophile antibodies are mainly in the alpha-2 and gamma globulin fractions of the serum protein. These experiments support the quantitative data of the cellulose acetate experiments and the results of the hemagglutination experiments with the separate protein fractions.

IV. Twelve mononucleosis sera and 12 normal sera were studied by immunoelectrophoresis in agar gel. In all 12 mononucleosis specimens, an abnormal pattern was observed in the alpha-2 globulin zone. One and sometimes two of the components showed precipitin arcs longer than in normal sera, extending into the beta-1 globulin zone. These arcs apparently represent one or more altered

components of the alpha-2 globulin, appearing on immunoelectrophoretic plates as a population of molecules with a wider than normal range of electrophoretic mobilities. These molecules could very possibly represent the heterophile antibodies shown by other methods to be present in the alpha-2 globulin.

A second observation not revealed by other methods was the fact that in nine of the 12 mononucleosis sera the beta-2M globulin arcs were more heavily stained and more distinct than in the normal sera used for comparison, when antibody against normal and mononucleosis serum was used. Since beta-2M globulin is a recognized antibody carrier, and in view of the observation made by Strannegard (45) that ox cell hemolysins migrated with the beta-2M globulin in mononucleosis sera, the above observation suggests that the increase in the gamma globulin fraction, observed in our electrophoretic analyses of 36 mononucleosis sera, was at least partly due to an increase in the beta-2M globulin, which is not distinguishable from the gamma globulin in electrophoresis on cellulose acetate.

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