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PROTEIN PROFILES OF BASIC HUMAN TEARS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Rodney Alan Hathaway, O.D., M.S.

* * * * *

The Ohio State University

1979

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ACKNOWLEDGMENTS

I am indebted to my advisor, Dr. Richard M. Hill, for his guidance and assistance throughout my professional and graduate studies, as well as during the course of this study.

My sincerest thanks go to Ms. Wendy Clark for her assistance in preparing the diagrams and photographs included in this document and to Mrs. Patricia Johnson for her technical assistance in preparing the manuscript.

My deepest gratitude is to my wife, Dr. Lucinda P. Hathaway, who devoted many hours to assisting in this investigation. Her encouragement and support are deeply appreciated.
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January 10, 1952 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . Born, Greenville, Ohio

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The precorneal tear film, forming a biophase between the external environment and the outer tissues of the eye, is of critical importance in maintaining maximum optical performance and the normal physiology of the anterior surface of the eye. Without adequate secretion and distribution of tears by the lacrimal system, corneal nutrition, transparency, and defense would be severely compromised. Because of its vital role in maintaining normal functioning of the eye, the chemical and physical properties of the tear film have been studied by many investigators.

Early investigations into the chemical nature of this fluid showed it to be composed primarily of water (approximately 98%), with the remainder (approximately 2%) being composed of organic and inorganic solids.\(^1\),\(^2\) Of this solid component, proteins were found to comprise the largest fraction.\(^3\) Because of their prominence, tear proteins have been the subject of numerous investigations.\(^4\)-\(^5\)

Although the early studies of tear proteins were directed primarily toward quantifying the amount of total protein present in normal tears, emphasis was later directed toward determining the protein fractions present within this total protein component. As instrumentation and technique advanced, the prominent role of tear proteins in maintaining tear film stability, corneal transparency, and defense of the eye became better understood.
A stable, precorneal tear film is required to provide an irregular corneal surface with a smooth optical surface for refraction to occur. The wettability of the cornea by the tear film results primarily from the presence and distribution of tear glycoproteins over the corneal epithelium. The surfactant nature of these glycoproteins aids in the transformation of a relatively hydrophobic epithelium into a wettable surface, thus allowing for the uniform spread of the aqueous phase of the tears.

Tear proteins also influence corneal hydration and transparency. Because of their large size, proteins do not pass freely in and out of the cornea, but, rather, remain in the tear film aiding in the establishment of an osmotic gradient between the tears and the corneal tissue. This gradient is further enhanced by the electrical charges associated with the protein molecules which attract ionic tear components (Gibbs-Donnan effect) yielding a tear film which is isotonic or slightly hypertonic with respect to the cornea. Although not as osmotically powerful as salt ions, this protein contribution to the tear osmotic force is, nevertheless, significant. Once established, this osmotic gradient influences the flow of water from the cornea into the tear film and assists the metabolic pumps of the cornea in regulating corneal hydration.

The electrically charged, ionic nature of the protein molecules provides the tear film the additional capacity of serving as a buffer system. Proteins are composed of amino acids bound together by peptide linkages. Some of these amino acids have free acidic radicals in the form of $-\text{COOH}$ which can dissociate into $-\text{COO}^-$ and $\text{H}^+$, while others have the basic free radical $\text{NH}_3\text{OH}$ which can dissociate into $-\text{NH}_3^+$ and $-\text{OH}^-$. As a result of their amphoteric nature, tear proteins can provide the
tear film with an assisting buffering system.

The defense of the eye provided by the tears results from the action of several individual tear protein fractions. Lysozyme and non-lysozyme acting factor (NLAF) have been shown to act quite effectively against certain foreign viral and bacterial contaminants. More recently, the isolation of immunoglobulins in the tear film implicates this fraction as an additional defense system active in the tears.

Recently, concentration determinations of tear proteins were shown to provide a valid indicator of the eye's response to various forms of stress, such as contact lens adaptation, drug therapy, and certain pathological conditions. Predictable changes in protein concentrations during such challenges may provide a useful tool for diagnosis in the future.

Thus, from their initial detection, the role of tear proteins in the normal functioning of the eye has become progressively better recognized. However, review of the literature reveals that significant limitations in our fundamental knowledge of tear protein profiles still exist. For example, while many have attempted to quantify total protein concentration in the tears, the values reported in the literature are quite varied. Much of this variability stems from the instrumentation employed, tear sampling techniques, and the type of tears collected (basic vs. reflex). Because of the large sample sizes previously required for protein analysis, most studies involved a "one shot" approach whereby tears were pooled or reflexly induced to obtain a single sample volume of sufficient size for
analysis. As a result, no reliable baseline information is available regarding diurnal variations of total protein concentration throughout the day, or from day-to-day for any one individual or for a normal population. Also lacking is information regarding baseline levels and diurnal variations of individual protein components.

For protein evaluations to prove useful in a prognostic sense, it is imperative that reliable baseline data be obtained. In this investigation, improved instrumentation and technique are employed to establish such baselines for the basic tears of a normal, healthy population.
The first investigation into the chemical nature of the precorneal tear film dates back to the late 18th century (Fourcroy and van Quelin, 1791). Interest in tear protein composition did not develop significantly until some time later. Possibly the first attempt to apply a systematic approach to studying protein was made by Magaard in 1882. In that investigation, Magaard collected tears from one individual and determined their water (98.12%), salt (0.416%), and organic component (1.463%) content. Using a nitric acid procedure, Magaard postulated that the organic precipitate resulting was protein.

Since that first study, other investigators, employing many techniques, have attempted to measure the tear protein concentration. v. Rotth (1922), using a refractometry technique on tears induced with ammonium vapor, found the concentration of total protein to range from 0.25 to 0.60 gm/100 ml. Ridley (1930), employing a salting-out technique to tears reflexly induced with lemon juice vapor, determined the amount of protein soluble in 50% ammonium sulfate, \( \text{(NH}_4\text{)}_2\text{SO}_4 \), and referred to this residue as albumin (0.345 gm/100 ml.). The remainder of the insoluble protein (0.275 gm/100 ml.), was classified as globulin, yielding a total tear protein concentration of 0.669 gm/100 ml.

In 1952, Jannola, employing a colorimetric technique (nephelometry), found total protein concentration to range from 0.136 to 0.592
gm/100 ml., with the average being 0.360 gm/100 ml. On the other hand, Balik et. al. (1933),\(^7\) employing a similar colorimetric technique, found total protein concentration to be approximately ten-fold higher (4.53 gm/100 ml.). In each of these studies, the tears analyzed were reflexly produced in response to a lacrimating agent.

By employing filter-paper electrophoresis, in conjunction with optical density determinations, Brunish (1957)\(^8\) found total protein concentration to range from 0.50 to 0.60 gm/100 ml. for tears stimulated with onion vapors. Using a modified form of electrophoresis whereby total protein was determined in bromphenol blue equivalents of lysozyme, Erickson (1958)\(^9\) reported a broader range of total protein concentration (0.39 to 2.90 gm/100 ml.).

In 1973, Tapaszto and Boross\(^10\) measured total protein in the tears of normal, healthy individuals with a Beckman\(^R\) spectrophotometer. The mean concentration of total protein found in this study ranged form 0.60 to 0.80 gm/100 ml.

More recently, Callender,\(^11\) Josephson,\(^12\) and McClellan\(^13\) have used a modification of the Lowry technique\(^52\) for protein determination, and found protein concentration levels comparable with those found by Erickson (1958) of 0.1 to 2.7 gm/100 ml.

Thus, it can be seen that over the years estimates of total protein concentration in the tears have resulted in a wide range of values (see Table 1). The results of each study appear to vary significantly depending upon the analytical method employed and the type of tears studied (normal vs. reflex).
While the initial investigations of tear proteins were directed toward studying this component as a homogeneous entity, interest soon developed in the quantitative separation of the individual protein fractions. Earlier studies of plasma protein using salt-fractionation, ultracentrifugation, and electrophoresis techniques demonstrated the presence of individual protein components forming the whole. Many of these techniques were soon applied to the tears as well to determine their component fractions.

When applied to the tears, methods of ultracentrifugation and salt-fractionation were found to provide little useful information. Although Ridley\textsuperscript{5} was somewhat successful in determining the albumin and globulin content of the tears using salt-fractionation, his application of this technique has since been criticized as not being able to sufficiently characterize proteins as individual chemical components.

In 1937, Tiselius developed the technique of electrophoresis which allowed for the separation of proteins in an electric field.\textsuperscript{53} Using this technique, various protein components of the serum at pH levels above and below their isoelectric points were shown to migrate various distances through an electrolytic medium when a current was applied to tear proteins.

In 1949, Svilens et al.\textsuperscript{14} attempted to fractionate tear protein into its components using free electrophoresis. This technique required placing 14 ml. of tears into a cellophane envelope and dialyzing them for 72 hours against a phosphate buffer, pH 7.9, with an ionic strength of 0.1. A current of 19 ma with a potential gradient of 10.8 volts/cm was then applied for separation to be completed. Four components were
found, three of which migrated toward the negative pole (cathode), while one moved toward the positive pole (anode). Further analysis of these fractions showed that the three positively charged components possessed lysozyme activity demonstrated by their ability to lyse Micrococcus Lysodeikticus. This property of tear lysozyme was previously demonstrated by Fleming (1922).  

The major difficulty in applying this electrophoretic technique to separate protein fractions was the large sample size (14 ml.) required for the analysis to be completed. Because of this large volume required, reflexly induced tears, using benzyl bromide as a lacrimator, were pooled from 215 individuals. Consequently, the results of this study clearly reflected a population average for reflex tears, rather than an individual analysis of the normal tear film.

Smolens and Leopold conducted a later study using a microelectrophoretic apparatus requiring smaller sample sizes and again found four components. This time, however, only one component containing lysozyme activity moved toward the cathode while three components not possessing this lytic ability moved toward the anode.

The 1950's brought forth the application of a different form of electrophoresis, filter-paper electrophoresis, to the study of tear protein fractions. In this procedure, filter paper served as the medium through which a buffered electrolytic solution passed when placed into an electric field. The simplicity and microcharacter (i.e. requiring small sample sizes for analysis) made this technique quite adaptable for tear protein evaluations.
In 1954, Caselli and Schumacher applied this technique to the tears collected from three subjects. Their results demonstrated the presence of 4 components in the tear film of each subject, three of which moved toward the anode, while a lysozyme fraction moved toward the cathode. Comparing the profiles of each subject, they noted that the size of each fraction varied among individuals, however, the relationship among the four components for each individual remained relatively constant for all subjects.

In 1955, McEvren and Kumura applied a modified form of filter-paper electrophoresis to determine the rate of movement of tear protein fractions with the hope of being able to chemically identify the protein fractions. Tears for this study were collected from the conjunctival sac using a 0.25 inch Whatman filter paper disc. The disc was then transferred to a 12 x 25 cm sheet of Whatman #1 filter paper, placing it toward the anode. Using a barbitol-NaCl buffer (pH 7.8, ionic strength 0.16), and an applied current of 6 ma., the protein samples separate into 3 fractions. The component traveling toward the negative pole with a mobility of $2 \times 10^{-5}$ cm/sec/volt/cm was determined to be lysozyme. The component moving rapidly toward the anode at a rate of $3.5 \times 10^{-5}$ cm/sec/volt/cm was noted to have a mobility similar to serum albumin which was used as a marker. The third group of proteins, lying in the region between the lysozyme and albumin components was thought to be an undifferentiated mixture of protein not sensitive enough for separation by this technique.

Until this time, lysozyme, by electrophoretic and Micrococcus Lyso- 
deikticus lysing studies, was the only tear protein fraction identified
with any certainty. The presence of albumin in the anodally attracted band was only suggested, while the chemical character of the middle band remained unspeculated. Of even greater mystery was the process by which these protein components entered the tear film.

In an attempt to answer these questions, Erickson (1956), applied a "hanging strip" technique of filter paper electrophoresis to tear samples. In this study, tears collected with Schirmer strips were placed onto a pre-buffered (phosphate buffer at pH 7.0, ionic strength of 0.2) filter paper strip. Across this strip, an electrical potential of 40 mA was applied for seven hours. With serum albumin used as a marker, Erickson noted that not only does the tear film separate into three fractions, but the component traveling anodally with serum albumin has a mobility slighter greater than serum albumin. This suggested a difference between tear albumin and serum albumin. To further investigate this difference, Erickson evaluated tears from an individual who previously had the accessory lacrimal gland of one eye surgically removed. Tears collected from this eye contained no lysozyme and only the slow moving serum albumin. The unoperated eye of this patient secreted the normal protein pattern. These results suggest that not only is the lacrimal gland solely responsible for secreting lysozyme, but it also either secretes something which alters serum albumin or secretes an albumin of its own.

In 1957, Erickson again applied this same electrophoretic technique and confirmed the difference between the faster moving tear albumin and serum albumin. She also noted the presence of a protein component in the central peak of the electrophoretic profile which had
the same mobility as serum beta globulin. From her studies, Erickson suggested that the normal tear protein profile reflects a 1:2:1 ratio of lysozyme:globulin:albumin. In addition, she postulated that half the albumin fraction was composed of the faster moving specific tear albumin with the other half being a product of serum albumin.

In 1997, Brunish set out to compare the normal (basic) tear protein profile to the profile of tears reflexly induced. The tears evaluated in this study included those induced by emotional stress, idiopathic excessive tearing, as well as those caused by irritants such as onion vapors or air pollutants. Employing a filter-paper electrophoretic technique, Brunish found that both emotional and excessive idiopathic tears showed similar profiles of 33.8% albumin, 8.8% globulin and 16.4% lysozyme. Irritant induced tears, on the other hand, showed albumin to be reduced to 19.3%, while the levels of globulin and lysozyme rose to 54% and 26.7%, respectively. The same number of fractional components, three, was noted in each form of tears.

By employing filter-paper electrophoresis with two buffer conditions (barbitol buffer, pH 7.8, ionic strength 0.1 and a sodium phosphate-\text{NaCl} (15%) buffer in 10% ethylene glycol, pH 7.0, ionic strength 0.07) and two electrical potentials, 3.5 V/cm and 3.75 or 4.16 V/cm, respectively, McEwen, Kimura, and Feeney (1958) successfully demonstrated the presence of 5 protein components present in the tear film. Two were easily identified as being tear albumin and lysozyme, while the remaining three components were designated as components I, II, III. The mobility of these three components appeared similar to serum globulins gamma, beta, and alpha₂, respectively. Staining properties, however, showed
differences from their serum counterparts.

The presence of three globulin fractions in the tears was confirmed by Erickson (1958) using filter-paper electrophoresis. Improvements in instrumentation, paper, and dyes (methanol soluable) over those previously used, as well as a change from phosphate to barbitol buffer, led to clearer separations and, thus, the identification of these components.

In 1961, Francois and Pabaey approached the study of tear proteins by employing an agar microelectrophoretic technique. Fractionation using this method was completed on a 12 Difco Noble agar plate using a veronal buffer solution (pH 8.4, ionic strength 0.05) and a potential gradient of 10 V/cm for 25 minutes. This method demonstrated the presence of four fractions, each having a relative mobility exceeding that of albumin with an additional fraction, lysozyme, which moved toward the anode rather than toward the cathode. The major problem encountered with this technique was the interaction of lysozyme with the agar which gradually formed a precipitate. It was believed that, due to this interaction, the mobility of lysozyme was changed, causing it to move toward the anode. Because of this interaction, the applicability of agar electrophoresis to tears has not been widely followed.

In 1965, Tapaszto and Voss employed a modification of this agar microelectrophoresis technique to tears collected both from healthy individuals and from individuals displaying signs of certain pathological disorders. Two changes in procedure included employing a different quality agar which did not interact with lysozyme and shifting the position of sample application more toward the cathode. The results of their study showed 5 protein groups. Of the 5 groups,
albumin, alpha globulin, and beta globulin moved toward the anode while lysozyme and gamma globulin migrated toward the cathode.

Within the 3 protein groups, 12 protein fractions, each having individual electrophoretic mobilities, were demonstrated. Two types of albumin were found. The faster moving tear albumin appeared in all samples, while serum albumin was detected only in tears of those subjects suffering from epiphora. Two alpha globulins, a fast and a slower component, were also noted. Although alpha_2 was noted in all samples, alpha_1, the faster component, was again noted only in patients suffering from epiphora. Beta globulin appeared in all samples and migrated to a position located between the site of sample application and the edge of the zone corresponding to alpha_2. The beta globulin fraction formed 3 component zones: beta_1, beta_2, and beta_3. More cathodally situated were three lysozyme bands and a gamma globulin zone, thus giving a total of 12 separable protein fractions.

In a later study by Tapaszto,^2 2 additional protein components were detected, increasing the number of separable protein fractions to 14.

The early 1960's brought about changes in the manner by which tear proteins were studied. In addition to the continued efforts put forth to more completely fractionate tear proteins, studies were initiated to identify and more completely characterize the chemical nature of the separated tear components. To do this, different investigative techniques were required.

In 1963, Chodirker and Tomasi^27 employed an immunoelectrophoresis procedure (a technique similar to micro agar electrophoresis) to study the immunoglobulin nature of tear gamma globulins. In this technique,
glass slides covered with a semisolid buffered agar (pH 8.2, ionic strength 0.025) served as the medium for the electrophoretic separation of proteins (Figure 1). A small hole cut into the agar served as an antigen well and was filled with tears. The glass slide was then placed across the baffles of an electrophoresis cell and a current of 4 mA was applied for a sufficient period of time (usually 90 minutes) for separation of protein components to occur. Once separation was complete, the antibody trough (a horizontal strip cut into the agar) was filled with antiserum to human tear components and allowed to incubate for 18 to 24 hours at room temperature. During this time, tears diffused into the agar and, when their protein fractions met migrating antibody, precipitin lines were formed. These lines were then stained and scanned using a densitometer.

Using this technique, Chodirker and Tomasi detected the presence of IgA and IgG in the gamma globulin region of the tear profile. The amount of IgA was then quantified using a radial gel diffusion technique.

In this technique, agar impregnated with a specific antigen directed against a single human immunoglobulin class was poured into a petri dish (Figure 2). After the agar hardened, wells (holes) were cut into the agar. Into one of these wells, a measured amount of tear fluid, usually 5 microliters, was placed. The antigen in the tears was then allowed to diffuse radially into the agar for a 24 to 48 hour interval. Where the antigen met the corresponding antibody in the agar, a circular precipitin ring was formed. The immunoglobulin level in the tears was then determined by comparing the ring diameter produced by the tear sample to the rings produced by serially diluted standard
solutions.

With this technique, Chodirker and Tomasi determined the concentration of IgA in the tears to be 7 mg/100 ml. The level of IgG could not be quantified with this procedure.

Over the years, numerous investigators have applied this technique, or modifications thereof, to accurately determine the levels of various immunoglobulins in the tears (Table 2). The results of those studies showed values for IgA to range from 7 mg % to 50 mg %, while IgG values ranged from 0 mg % to 79 mg %. In two studies, IgM was detected, but occurred in low concentrations ranging from 0 to 5 mg %.

To date, the presence of IgD has not been detected in the tears.

With one exception, all studies showed a higher level of IgA in the tears than IgG. This is contrary to levels seen in human serum where IgG occurs in a concentration 8 to 10 fold greater than IgA (serum IgG = 1335 mg/100 ml, serum IgA = 178 mg/100 ml). This finding tends to indicate that tear IgA is not merely a serum filtrate.

Instead, its presence in the tear film might result from special transport mechanisms or local production by secreting glands. The identification of an additional "secretory piece" present in tear and parotid IgA, but absent from serum IgA, further supports this idea of selective transport or local production of tear IgA.

To date, the exact mechanism by which IgA gains entry into the tear fluid remains unknown. However, two theories have been proposed. Brambel et al. has suggested that individual IgA molecules have an active carrier site which acts in their selective transport from the serum into certain body secretions. Pierce, on the other hand,
proposes the lymphatic system as the ultimate source for tear immunoglobulins whereby conjunctival lymph nodes produce and secrete tear immunoglobulins. Which, if either, theory is correct remains uncertain even today.

In 1964, Josephson and Lockwood confirmed the results of Chodirker and Tomasi and identified 3 additional components in the tear profile. In their investigation, both antiserum to total tears (produced by repeatedly injecting rabbits with human tear protein solutions) and specific antisera commercially available were used. Immunoelectrophoresis carried out in the manner previously described yielded 4 to 8 precipitin lines, depending on the particular eluate used and the type of tears studied (basic vs. reflex).

In normal, non-reflexly induced tears, specific tear albumin and trace amounts of serum albumin were demonstrated. Two glycoproteins, oroseromucin and ceruloplasmin, as well as tear IgA, were also noted to be present.

When the eye was traumatized, however, the amount of serum albumin and gamma globulin in the reflex tears were seen to increase. In addition, the trauma induced tears were shown to contain small quantities of an additional glycoprotein, transferrin.

While the role of IgA in the tears is most likely that of serving as an antibody in the defense of the eye, the function of oroseromucin, ceruloplasmin and transferrin is less defined.

Chemically, oroseromucin is a glycoprotein of relatively low molecular weight (41,000) and has an electrophoretic mobility similar to alpha1 globulin. Although its functional role in serum is
unknown in tears, it is believed to increase the wettability of the corneal epithelium.\textsuperscript{41}

Ceruloplasmin, like oroseromucin, is a glycoprotein, but of relatively high molecular weight (151,000).\textsuperscript{55} The electrophoretic mobility of this component is similar to \( \alpha_2 \) globulin. Although the copper-binding property of this protein suggests its role in copper metabolism, recent evidence suggests that this protein has powerful oxidizing properties which may more likely account for its presence in the tear film.

The third glycoprotein, transferrin, is an iron-carrying protein having an electrophoretic mobility similar to \( \beta_1 \) globulin.\textsuperscript{55} Like serum albumin, this protein increases in concentration only after insult to ocular tissue has occurred. Recently, the presence and physiologic role of transferrin in the tear film has been questioned by Broekhuyse (1976).\textsuperscript{40} By employing monospecific antiserum against lactoferrin, immunoelectrophoresis and immunochromatographic techniques confirmed that lactoferrin, not transferrin, is present in the lacrimal tear fluid. The strong metal-binding properties of this protein suggest that it serves to inhibit the growth of microorganisms and, as such, may act together with immunoglobulins, lysozyme, and NLA\textsuperscript{42} in protecting the eye against infection.

In 1968, Josephson and Weiner,\textsuperscript{12} again employing an immunoelectrophoresis procedure, noted the presence of tear pre-albumin, a protein unique to the tears. In 1968, Sapse, Bonavida, and Stone\textsuperscript{43} confirmed the presence of this protein and emphasized its uniqueness by calling it "specific tear pre-albumin." Until this time, all proteins found in
the tears were either identical to those found in serum, such as ceruloplasmin, or were secreted proteins, similar to, but not identical with, their serum counterparts such as tear IgA with its additional secretory piece. Specific tear pre-albumin had no serum counterpart.

The 1970's brought forth further studies to detect and quantify immunoglobulin concentration in the tears. In addition, the concentration of total protein and certain individual tear protein components began to be evaluated as indicators of the eye's response to stressful situations such as contact lens adaptation, drug therapy, or certain disease states.

In the initial period of contact lens adaptation, the cornea swells. This change in corneal thickness results either from a disturbance in corneal metabolism usually associated with oxygen deficiency, or from changes in tear tonicity. The tonicity of the tear film is regulated by both inorganic constituents (salts) and organic components such as protein. Any change in concentration of either of these component groups could alter the osmotic balance between the corneal tissues and the tear film in accordance with the Gibbs-Donnan effect. Such alteration could lead to corneal swelling.

The effects of contact lens adaptation on the total tear protein concentration were first examined by Uniacke and Hill in 1969 and later by Callendar and Morrison in 1974. Both groups noted a significant decrease in total protein concentration during the adaptation process occurring simultaneously with corneal edema. Once full adaptation was achieved, protein levels usually returned to their pre-adaptation levels and corneal swelling disappeared. This finding
suggested that protein concentration may serve useful as an indicator in monitoring the rate of adaptation to hard contact lens wear.

More recently (1976), the level of one protein component, lysozyme, was shown to be a useful indicator of ocular toxicity produced during treatment with proctolol. Proctolol is a beta-adrenergic receptor blocking agent used in the management of cardiac arrhythmia and ischaemic heart disease. Continued use of this drug presents, in some patients, symptoms of dry eyes with subconjunctival fibrosis and occasional corneal ulceration. Mackie (1977) noted that patients exhibiting these signs and symptoms usually have a reduced tear lysozyme concentration. The idea that alteration in tear lysozyme concentration may account for the signs and symptoms noted is further supported by earlier studies, whereby a reduction or absence of tear lysozyme was noted in patients suffering from keratoconjunctivitis sicca, Sjogren's syndrome, or peripinal ring keratitis, who displayed similar signs and symptoms. In this study, Mackie suggested that the reduced lysozyme results from the effects of the beta-blocking drug on the control of the lacrimal gland, inhibiting its release of lysozyme. By monitoring tear lysozyme levels of patients undergoing proctolol treatment, any changes in concentration should serve as an indicator for changing therapy in order to prevent subsequent ocular complications.

From reviewing the literature, it becomes evident that our understanding of tear proteins has developed significantly over the years. From their initial detection, much has been learned regarding their chemical identity and functional role in support of the eye. Missing, however, is a valid understanding of the concentration of these
protein components among individuals in a normal population. Although many attempts have been made to determine protein levels, the results are quite variable. Such discrepancies may be attributed to method of analysis employed, or the type of tears studied.

Unlike plasma, tears are not readily available in large quantities. Previous attempts to quantify tear protein concentration have involved employing techniques traditionally used for plasma studies, and, as such, have required large sample volumes. To achieve sample volumes of suitable size for analysis, tears were either pooled or reflexly induced using lacrimating agents. In either case, no reliable baseline total protein concentration levels could be determined for basic tears.

Recently developed instrumentation now makes it possible to evaluate protein concentration in sample volumes physiologically attainable without the need for inducing reflex tear flow. By employing this technique, it should now become possible to attain reliable baseline concentration levels for total protein concentration in basic tears. By combining this technique with cellulose acetate electrophoresis evaluations of tear protein components, it should be possible to sufficiently quantify and determine baseline concentrations of these components as well.
OBJECTIVES

Due to the many inconsistencies regarding tear protein concentration levels now present in the literature, the following objectives will be investigated in this study.

1. To determine the mean concentration of total protein in basic tears as well as the range of values existing in a normal population.

2. To investigate diurnal changes in total protein concentration and search for any predictable patterns within a given individual or within the population as a whole.

3. To determine the mean concentration of several individual tear components (lysozyme, globulin, albumin) in the tears of individuals or for a normal population.

4. To investigate the presence of any predictable diurnal changes in the concentration of each protein component.

5. To determine what alterations occur in tear protein concentration when the eye adapts to hydrophilic contact lens wear or undergoes other forms of mechanical irritation.
METHODOLOGY

INTRODUCTION

In this investigation, attempts are made to circumvent some of the problems of earlier investigations and to determine individual profiles for total protein and several separable protein fractions. Important to a valid investigation of these components is the development and application of controlled tear collection techniques together with instrumentation which can be used to accurately analyze minimum basic tear volumes.

Only subjects able to contribute basic tears (i.e. without inducing reflex tearing) were selected. Instrumentation requiring a small sample size for analysis (5 microliters) was used to determine total protein and an electrophoretic method requiring 2 microliters was applied to fractionate total protein into three subcomponent fractions.

SUBJECT SELECTION

Subjects participating in this study were selected from patients examined at The Ohio State University Optometry Clinic. Candidates were required to meet several screening criterion. Only healthy individuals, not presently wearing contact lenses or taking any medication, were considered. Each candidate was required to demonstrate the ability to produce and collect basic tears of suitable sample size for analysis within a five minute collecting period. The minimum acceptable volume was 7 microliters. Since one of the objectives of this investigation was to
detect and monitor cyclic changes in total protein concentration, only subjects having a sufficiently liberal daily schedule which would allow them time to collect tear samples hourly for five consecutive days were considered.

An additional requirement of four subjects selected was they have significant refractive error to warrant correction with hydrophilic lenses. This requirement made it possible to determine whether adaptation changes occur similar to those known to occur during adaptation to conventional, PMMA contact lenses.

Eleven subjects, 8 male and 3 female, ranging in age from 16 to 59 years, were ultimately selected for this investigation. Of these subjects, four, one male and three female, were eventually fitted with hydrophilic lenses.

All subjects occupied similar environments, being in air-conditioned work or classroom atmospheres during the day and air-conditioned home environments during the evening hours. None were knowingly exposed to any industrial gasses or unusually high levels of environmental contamination. Each signed The Ohio State University Human Subject Consent Form PA-028 (see Appendix A).

COLLECTION OF TEAR SAMPLES

Subjects participating in this investigation were required to collect 12 to 14 consecutive hourly tear samples from 8:00 a.m. to 10:00 p.m. for five consecutive weekdays. Weekdays were chosen due to the regularity of most subjects' schedules during the week.

Microcapillary tubes of a 25 microliter volume were used to collect and store the tear samples. Each subject was given a vial of sterile
"microcap" capillary tubes and instructed on the proper technique for removing the tubes from the vial to avoid contamination of the sampling end of the tube. Each was then instructed to collect tear samples by gently pulling the lower lid away from the eye and placing the sterile end of the capillary into the lower conjunctival cul-de-sac area. The tube was held in this position until an adequate volume of tears (seven to nine microliters) flowed up the tube by capillary attraction. Once a suitable volume of tears was collected (approximately one-third of the tube filled), the ends of the capillary were immediately sealed with Critoseal, a silicon clay, to prevent spillage or evaporation. Each tube was then labelled with the date, time of day, and the subject's initials.

During the collection procedure, each subject was cautioned to avoid mechanical irritation of the conjunctival tissue with the end of the capillary which would stimulate abnormal (reflex) tear flow. As an additional control, to help ensure that tears analyzed were normal, basic tears, each of the eleven subjects was required to develop and demonstrate his collecting skills by taking hourly samples for three days prior to the beginning of the investigation.

During the course of this study, the subjects delivered their tear samples to the laboratory at the end of each day. Efforts were made to analyze all samples for total protein concentration within 16 hours after being collected in order to minimize effects of protein deterioration due to aging. Separate diet sheets were completed and submitted with the samples collected each day.
ADDITIONAL TEAR SAMPLES REQUIRED

All subjects were requested to collect hourly samples (nine microliters in volume) for an additional day in order that both total protein and the relative amount of individual protein components be determined.

Four subjects adapting to hydrophilic lens wear were requested to submit tear samples for an additional seven days. In addition to the samples taken for six days prior to lens adaptation, each individual was asked to submit nine microliter samples for five days during lens adaptation and again for two days after adaptation was complete.

INSTRUMENTATION: TOTAL PROTEIN

The concentration of total protein present in each tear sample was determined using an Abbott ABA-50 \textsuperscript{R} Bichromatic Spectrophotometer (Figure 3). Recent in design,\textsuperscript{56} this semi-automated clinical chemistry system was developed primarily for analysis of chemical components in plasma and blood serum. It is applied in this investigation to determine total protein concentration in human tears.

Basically, this system works by providing both a stable environment for a color developing chemical reaction to occur and controlled conditions for measuring the extent of the chemical reaction spectrophotometrically.

The chemical reaction used for determining the concentration of total protein present in the tear samples is a biuret reaction whereby protein reacts with a substrate reagent (Total Protein A-Gent\textsuperscript{R}), causing a chromogen in the substrate to develop. In this particular technique,
the biuret reaction is based upon the production of a purple color when tear proteins containing peptide bonds are mixed in the alkaline medium of the substrate. The purple color resulting is due to the formation of coordination complex between the cupric ion of the substrate and the protein nitrogen under constant temperature conditions. The amount of chromogen developed is proportional to the concentration of the protein present in the tear sample. (See Appendix B for detailed description.)

The chemical reaction of protein and substrate takes place in clear plastic cells, each having the same dimensions, being serially arranged in a circular fashion to form a multicuvette of 31 cells (Figure 4). To maintain a controlled temperature environment, the multicuvette is placed into a thermostatically controlled water bath maintained at 37°C.

Into each cell in the multicuvette, 250 microliters of substrate is dispensed using a calibrated SMI Micro/Pettor^ micropipette (accuracy ± 5.2 microliters). After a five minute interval, the temperature of this substrate equilibrates to the temperature of the water bath and is ready for the reaction with protein to take place.

Sealed microcapillaries containing hourly samples are analyzed for total protein by first removing both ends of the tubes using a glass file. An SMI Micro/Pettor^ micropipette, previously calibrated to contain 5 microliters of fluid (accuracy ± 0.138 microliters) is placed flush against the end of the tube and used to extract a measured volume of tears (Figure 5). The contents of the micropipette are then expelled into a multicuvette cell containing substrate. The solution is then gently stirred several times with the end of the capillary and allowed to stand for five minutes to allow the chromogen to develop.
At the end of this time period, the protein concentration is determined using the built-in spectrophotometer system.

Measurements of protein concentration, using this system, are accomplished by directing the light via prisms and lenses from a tungsten halogen source through a multisegment filter wheel, which in turn produces light at two wavelengths (Figure 6). One wavelength is chosen close to the absorption peak ($\lambda_p$) for the chromogen, while the other wavelength ($\lambda_s$) is off to the side of the peak. As the filter wheel spins, these wavelengths alternately pass through the colored solution contained in a single cell of the multicuvette positioned on the optical axis of the system. Thus both wavelengths pass through the same optical path. The light transmitted through the cell is then directed by a prism and system of lenses to a photomultiplier tube. A photoelectric device detects which filter segment ($\lambda_p$ or $\lambda_s$) is in the light path at each instant and switches the photomultiplier output to one of two measurement channels. A digital converter processes the intensity signal of the transmitted light at each wavelength ($\lambda_p$, $\lambda_s$) and calculates the absorbance. The absorbance value is then displayed on a visual readout in terms of the concentration of protein present in the sample.

Prior to the analysis of tear samples, the instrument was calibrated using known concentrations of protein solutions formulated from Total Protein Standard™, commercially available from Scientific Products, Dade Division. (See Appendix C for chemical characteristics.) The linearity of the instrument was confirmed using standard control solutions ranging in concentration from 0.125 to 8.20 gm/dL (Appendix D).
INSTRUMENTATION: PROTEIN FRACTIONATION

Electrophoresis is defined as the movement of colloid particles in an electric field. In the electric field, various proteins show different migration rates based upon their individual differences in electrical charge, shape, and size (molecular weight). The pH of the buffer also plays a critical role in regard to the type (positive or negative) and the number of charges on each colloid protein molecule. In zone (moving boundary) electrophoresis, the charged particles are placed on a stabilized medium which serves to contain the separated protein zones after migration is completed. In this investigation, a Beckman R-101 Microzone\textsuperscript{R} electrophoresis system, employing a cellulose acetate membrane as the stabilizing medium, is used for separating tear proteins (Figure 7).

In theory, the cellulose acetate membrane does not interact with the protein. Each membrane is composed of biologically inert cellulose acetate material, 130 microns thick, containing uniform pores less than 2 microns in diameter. The membrane is very porous, permitting free but controlled flow of buffer solution from one reservoir to the other. Because of this nature, movement through the membrane when current is applied can be related almost exclusively to the electrophoretic mobility of the proteins. Thus, clear fractionation of individual components can be accomplished with minimal sample loss.

After subjects collected samples hourly for 5 consecutive days for total protein determinations, they were asked to take samples (nine microliters in volume) for an additional day so that the amount of each of three separable protein fractions could be determined. Five
microliters from each sample were required for total protein analysis, while two microliters were required for electrophoresis. The two microliters remaining served as a reserve in the event that part of a sample was lost during the transfer process.

Using a micropipette, two microliters of tears from each capillary were extracted and placed into separated channels on a prebuffered cellulose acetate membrane mounted in a Beckman R-101 Microzone\textsuperscript{R} electrophoresis cell. Beckman B-2\textsuperscript{R} buffer, a diethyl barbituric acid-sodium diethyl barbiturate combination with an ionic strength of 0.75 at pH 8.6 was used as the vehicle. Across this membrane a constant potential of 3.5 milliamperes and 250 volts was passed for twenty-five minutes, allowing the albumin (with alpha-globulin included), gamma globulin, and lysozyme to be separated into three main bands (Figure 8).

After separation was completed, each membrane was placed into a solution of Beckman Fixative-Protein-Dye\textsuperscript{R}, composed of 0.5 grams Ponceau stain, 7.5 grams trichloroacetic acid, and 7.5 grams salicylic acid, in order to stain the protein bands. After a ten minute interval, the membrane was transferred to a rinsing tray where successive rinsings with 5% acetic acid were completed until the membrane appeared white with only darkly stained protein bands remaining. Next, the membrane was gently agitated in a tray containing 100 ml of 95% ethanol to remove any water present in the membrane, then transferred to a tray containing a clearing solution (25 ml acetic acid and 75 ml 95% ethanol). Agitation in this solution for 60 seconds allowed for the transformation of the originally white, opaque membrane into a transparent membrane containing darkly stained red protein bands. Finally, the membrane was
removed from this solution on a glass plate and allowed to air dry overnight before being mounted in a clear cellophane envelope. The relative amount of protein present in each band was then determined by scanning each membrane with the Beckman Microzone™ densitometer (Figure 9).

Identification of components present in each band was determined by comparing migration characteristics of tear component proteins to those of known standards of human serum albumin, alpha globulin, gamma globulin, and hen's egg lysozyme (Figure 10). (See Appendix C for chemical purity of each standard.)

To determine the effects of hydrophilic lens adaptation and subsequent wear on total tear protein concentration, four subjects were asked to take hourly samples for five additional days while adapting to their lenses. During this adaptation period, lenses were worn only five hours each day, from 11:00 a.m. to 4:00 p.m. The 11:00 sample was taken immediately after lens insertion, while the 4:00 sample was taken immediately after lens removal. Once full time wear was established (usually after an additional week of adaptation), tear samples were taken hourly for two additional days during full time lens wear.
RESULTS

This study consisted of analyzing total protein concentration in tear samples collected hourly over a five-day interval by eleven subjects. The number of samples analyzed from each subject ranged from 55 to 75. The mean number of samples per subject was 67. Variation in the number of samples from each subject resulted from missing samples, rejected samples, or loss of samples during the analysis procedure. Missing samples resulted primarily from the subject's inability to collect a sample during a particular hour of the day. Rejection of samples was based upon the presence of numerous bubbles, or clumps of cells or solidified tear components visibly detected in the capillary tubes. In addition, some samples were rejected when the volume of tears collected was insufficient for analysis. A small number of samples were lost during analysis due to procedural error.

Graphs showing the hourly tear protein concentration for each subject over the five-day sampling period are seen in Figure 11 (a-k). These graphs display the basic data that this paper develops. From them, it can be seen that tear protein concentrations can vary rather dramatically from hour to hour, or from day to day for any given individual. By comparing the graphs of each subject, it is clear that much variability exists even among individuals within this sample population.
As a means of viewing the absolute value of total protein concentration in the tears, a frequency histogram with an overlaid normal curve was compiled for each subject in Figure 12 (a-k). Examination of these curves show a wide range of individual mean total protein levels among the subjects. These values range from a mean of 0.56 gm/100 ml (s = 0.11) to a mean of 1.11 gm/100 ml (s = 0.46), a difference of 0.55 gm/100 ml (see Table 3).

Figure 13 shows a frequency histogram for the entire population. For this population, the mean value for total protein concentration was found to be 0.81 gm/100 ml with a standard deviation of 0.25 gm/100 ml for the 732 samples analyzed. Visual examination of this figure reveals that a symmetrical distribution of protein concentrations does not exist. While the range of individual protein determinations runs from 0.41 to 2.00 gm/100 ml, 84% of these determinations fall within the range of 0.40 to 0.99 gm/100 ml. (see Table 4). This distribution indicates a skewedness of the data showing a significant degree of leptokurtosis.

This frequency profile can be more fully delineated by specifying several of its statistical variables. For this distribution, the second moment, \( m_2 \), is 0.06; the third moment, \( m_3 \), is 0.03; and the fourth moment, \( m_4 \), is 0.03. The moment coefficient of skewness for this distribution, \( \gamma_1 \), is 1.77, indicating positive skewedness (for symmetrical curves, \( \gamma_1 = 0 \)). Since a normal curve distribution has a moment coefficient of kurtosis (\( \gamma_2 = 3 \)), the moment coefficient of kurtosis found in this study, \( \gamma_2 = 7.83 \), indicates a significant amount of leptokurtosis of the data collected (see Appendix E).
For this sample population, the median value for total protein concentration is 0.76 gm/100 ml, with a mode value of 0.73 gm/100 ml.

To detect the presence of any predictable cyclic trends occurring in total protein concentration throughout the day, the five daily readings for each hourly period were summed together for each individual and the mean total protein concentration for each hour was determined. These values, as well as the high and low readings (range limits) are plotted in Figure 14 (a-k). Also indicated in this figure are the time periods during which meals were eaten.

In general, these curves appear to oscillate very actively about the over-all (5 day) mean value determined for each individual. The number of oscillations and the amplitude of each about the mean during the day appears to vary among the subjects. No predictable cyclic pattern is seen in relation to the times of meal intake.

To further explore the data for the presence of any repeatable cyclic variations in total protein concentration which may have been masked by examining only the mean values, the hourly total protein concentration levels measured each day were plotted and examined separately. The daily profiles for one individual are seen in Figure 15 (a-c). Again, it is evident that the protein concentration levels tend to oscillate, in most instances, about the mean protein concentration for each day. The number of oscillations varies from day to day.

To more fully examine the data for the presence of any repeatable cyclic pattern, computer analysis was performed using the following function: 

\[ y = \text{intercept} + \beta_1 \sin \left( \frac{2\pi T}{P} \right) + \beta_2 \cos \left( \frac{2\pi T}{P} \right) \]
Various time intervals (P), starting at different hours of the day (T), were examined. The time intervals studied included: P = 1, 2, 3, and 4-hour blocks beginning from each hour of the sampling day (T = 8 a.m. to 8 p.m.). Using this function, no predictable cycles could be found in the daily profiles of each individual subject.

To determine whether a predictable cycle occurs within the sample population, the five daily values for each of the eleven subjects were summed together for each hour and their mean values and standard deviations were determined and plotted (Figure 16). The relatively flat nature of this curve demonstrates the lack of any predictable cyclic variation in total protein concentration among the population studied.

For an additional day, all subjects were asked to collect hourly tear samples so that the relative amount of individual protein fractions could be determined. The electrophoresis technique employed in this study was capable of separating total protein into three fractions: I, II, III. By comparing the zones of the separated tear components to those produced by standard control solutions, the individual protein components present in each band were determined. Fraction I moved toward the cathode with a mobility similar to lysozyme. Fraction II demonstrated a mobility similar to globulin, while Fraction III moved toward the anode and was determined to be composed primarily of albumin with some alpha globulin included.

Graphs showing the percentage amounts of each of the three separable fractions are plotted for each individual in Figure 17 (a-k). From these, it is seen that the ratio among the three protein
components does not remain constant throughout the day, but, rather, undergoes rather dramatic fluctuations.

To further explore the changes in proportion of each fraction and determine the possibility of two fractions changing together in a predictable pattern rather than each changing independently, a binomial non-parametrical statistical procedure was employed to compare fractional pairs testing the following hypothesis:

\[ H_0: \text{the fractions vary by chance.} \]

\[ H_1: \text{the two fractions do not vary by chance.} \]

The criteria of whether two fractions vary together in the same direction is based upon their number of matches \((X)\). To determine the number of matches occurring, each protein fraction profile was examined in terms of whether its proportion increased or decreased during each hour of the day. If the proportion increased a (+) value was assigned to the change, while if a decrease was noted, a (-) value was assigned. If the level remained constant for two consecutive hours, a (±) value was assigned (see Table 6).

When comparing each of the three fractions to one another, the number of matches were determined.

Under \( H_0 \): \( p \text{ (matches) } = \frac{1}{2} \)

Under \( H_1 \): \( p \text{ (matches) } \neq \frac{1}{2} \)

Three possible conclusions can be made. If the number of matches \((X)\) is average, we accept \( H_0 \) and the two fractions being compared move together only by chance. If the number of matches is very high, \( H_0 \) would be rejected and the two fractions being compared would be seen to move in the same direction. If the number of matches was low, \( H_0 \) would
be rejected and the two fractions being compared would be seen to move oppositely.

Due to the small number of samples examined for each subject, it became impossible to evaluate this hypothesis for each subject at a significance level of 0.05. The alpha levels used ranged from 0.02 to 0.10, varying with the number of samples analyzed for each subject (see Appendix E).

The results for each individual subject are shown in Table 7. For the sample population of eleven subjects, four subjects showed that Fraction I and Fraction III vary together, but in opposite directions. The remaining seven subjects showed these fractions to vary by chance. For five subjects, Fraction II and Fraction III were also found to vary inversely, while for one subject, these fractions varied together. The remaining five subjects showed these fractions to vary by chance. When Fraction I was compared to Fraction III, all eleven subjects showed these components to vary by chance. These findings indicate no consistent correlative variations exist among these fractions for the subjects examined.

The number of hourly tear samples analyzed for each subject ranged from 6 to 14 with a mean of 11 samples per subject. By summing the values for each fraction and determining the mean and standard deviation, it is clear that the ratio of these components is not fixed, but varies from individual to individual (Table 9). The over-all mean percentage ratio for the entire population is

\[
\text{Fraction I : Fraction II : Fraction III}
\]

\[
21.7\% : 43.6\% : 34.8\%
\]
For this population, the percentage values for Fraction I range from 16% to 38%, while those for Fraction II range from 34% to 53%. Fraction III is seen to have an even broader range, from 21% to 50%.

To determine the relative amount of protein present in each band, the total protein concentration for each sample was multiplied by the proportion of each protein fraction occurring within the sample. Both the total protein and the amount of protein present in each fraction for each subject are graphed in Figure 18 (a-k).

Visual examination of these graphs again shows considerable variation in protein concentration throughout the day. To examine the relationship of changes in individual protein components with changes in total protein concentration, a Friedman Ranking Test was applied, whereby the magnitude of change in total protein concentration was compared to the magnitude of change occurring in the protein concentration for each individual fraction (see Table II).

Of the eleven subjects considered, seven showed the variations in total protein concentration to be most highly correlated with changes in the concentration level of Fraction II (significance 0.05). Three showed the highest correlation with changes in Fraction III (significance 0.10). One showed equal effects from Fractions II and III.

The mean and standard deviation for the amount of protein present in each fraction for each individual are given in Table 3. The average concentration values for each fraction seen in the entire population are: Fraction I, $\bar{x} = 0.19 \text{ gm/100 ml.}$ ($\sigma = 0.06 \text{ gm/100 ml.}$), Fraction II, $\bar{x} = 0.39 \text{ gm/100 ml.}$ ($\sigma = 0.19 \text{ gm/100 ml.}$), and Fraction III, $\bar{x} = 0.31 \text{ gm/100 ml.}$ ($\sigma = 0.12 \text{ gm/100 ml.}$).
To examine the effects of hydrophilic lens adaptation on the concentration of protein in the tears, tear samples were collected by four subjects for an additional seven days. During the five-day adaptation period, lenses were worn only 5 hours per day, from 11 a.m. to 4 p.m. To monitor changes in protein concentration during adaptation, the mean concentration of total protein during the hours of contact lens wear was compared to the mean total protein concentration present during the same time interval for the week prior to lens wear (Figure 19 (a-d)). The number of samples analyzed for each subject during the adaptation period ranged from 27 to 30 (see Table 10).

For each subject, a reduction in mean total protein concentration was seen during this adaptation period. Subject I had a decrease of 6.85% in mean total protein concentration. Subject II had a decrease of 4.82%. Subject III displayed a reduction of 2.63%, while subject IV had the largest decrease, 10.71%.

For most subjects, the lowest mean concentration was noted to occur during the first hour after lens insertion, presumably resulting from the reflex tearing produced by the mild mechanical irritation of the eye caused by the lens. After the first hour, the levels of protein tended to increase with increased wearing time, usually approaching the preadaptation level. After full-time wear was achieved (i.e. 12 to 14 hours), the mean tear protein concentration level varied little from its preadaptation level.

Although the mean values of total protein appear to change during the adaptation period, a one-way analysis of variance (α = 0.05) showed no significant changes in total protein concentration during the
adaptation period or during the post-adaptation phase.

To determine the effect of mechanical irritation on the levels of protein in the tears, one subject was asked to remain at the laboratory for one full day so that both normal and reflexly induced tears could be collected and analyzed. During the day, a repeatable cycle of tear samples were collected every two hours. In this cycle, the subject was asked to first collect a sample of normal, basic tears. The eye was then irritated by rubbing the lower conjunctival lid area with a moistened cotton swab. The reflexly induced tears were then collected. After a two-hour interval (a time interval allowed for eliminating any residual effects of reflex tearing), the cycle was repeated. The differences in protein concentration for total protein as well as the changes in the individual protein fractions are seen in Table 11. During the course of the day, the mean tear profile for tears reflected a ratio of:

\[
\]

When reflexly stimulated the ratio changed to 24% : 25% : 51%.

During reflex stimulation, total protein concentration had a mean decrease of 23%, while Fraction I increased 13%, Fraction II decreased 17%, and Fraction III increased 15%. 
DISCUSSION

Although the presence of protein in the tears has been known for over 100 years, attempts to determine its concentration have yielded variable results stemming primarily from the methods of analysis employed and the type of tears analyzed (pooled or reflexly induced). As a result, no reliable baseline information regarding total protein as it normally occurs in basic physiological tears became available.

The instrumentation used in this study offers distinct advantages over previous types in that it is capable of accurately analyzing total protein concentration in small sample volumes (5 to 10 microliters), thus eliminating the need for pooling or using lacrimating agents. The Abbott Bichromatic Analyzer 50R is among the most reliable systems available today for measuring proteins in solution. The use of the biuret reaction has been proven to be a highly specific and reliable test for measuring protein concentration. The distinct advantages of a bichromatic photometry system for measuring the extent of color endpoint reactions over the earlier used monochromatic techniques have been previously discussed (Appendix B). In this investigation, the reliability and accuracy of this system for measuring total protein concentrations were demonstrated by making repeated measurements on standard solutions of known concentrations (Appendix D).

By employing this technique to measure human tear protein concentration, this study offers several exceptional features over previous studies. Because of the small sample volume required, this
investigation represents the only study of basic tear protein. As an additional control to ensure that the tears analyzed were basic tears, only subjects demonstrating the ability to produce and collect tears without inducing reflex tearing were selected.

The small sample volume requirement offered the additional advantage of making it possible to monitor tear protein concentration throughout the day, i.e. by allowing multiple sampling, an approach not previously attempted.

The total number of samples analyzed in this study, 732, is, at present, the largest sample size (N) examined for determining mean protein concentration. The number of subjects, 11, studied on an hourly basis provides the most comprehensive study of tear proteins in a sample population.

The over-all mean total protein concentration for this sample population is 0.81 gm/100 ml. (range 0.56 to 1.11 gm/100 ml.). This value is somewhat higher than those reported earlier by v. Rotth (0.25 to 0.6), Ridgley (0.669), Junnola (0.136 to 0.596), and Brunish (0.40 to 0.60). Such discrepancies most likely result from the reflex nature of the tears previously examined. Studies have shown that total protein concentration decreases exponentially with increased rate of tear flow. Even in this investigation, it was noted that the reflex tearing occurring during the mild mechanical irritation produced by hydrophilic lens adaptation can cause a mean reduction in total protein from 2 to 10 percent. The more severe mechanical irritation caused by rubbing the conjunctiva with a cotton-tipped applicator produced an even greater decrease in mean total protein
concentration of 23 percent.

Of interest is the wide range of mean total protein concentrations present within this sample population, ranging from a mean of 0.56 to 1.10 gm/100 ml. Prior to the start of this investigation, each subject was given a comprehensive eye examination. Each subject selected was free of any form of ocular or systemic disorder. During the course of this investigation, all subjects remained in good health. This wide range in mean total protein concentration, therefore, reflects values which can be found within a healthy population. It is remarkable to note that, even with such a large range in protein concentrations among these subjects, the vital role of tear proteins in the defense and physiological support of the eye appears uncompromised.

By examining total protein concentration hourly over a five-day interval, it can be seen that not only do the levels of protein vary from day-to-day, but that dramatic changes may also occur from hour-to-hour. Both visual examination of the profiles, as well as computer analysis, failed to show any repeatable cyclic pattern in the mean concentration levels throughout the day for any one individual or for the total population. It, therefore, appears that if there exists a control system for regulating protein concentration in the tear film, the system has a wide operating range and does not appear to regulate any repeatable cycle.

To further assure that a predictable pattern was not masked by examining only the mean hourly levels, the individual daily profiles were plotted for each individual. Again, no predictable pattern could be seen. There did not appear to be any repeatable cycles which were
merely shifted in phase from day-to-day. The number of cycles present each day, as well as their amplitudes, appeared quite variable.

Although previous investigations have shown that a variation in concentration of certain tear components, such as glucose and calcium, could be correlated with dietary intake, no such predictable changes in tear total protein concentration appear related to diet. This most likely results from the fact that dietary proteins, unlike many other metabolites, require a considerable length of time to be broken down by the digestive system into usable amino acids. Protein digestion and absorption usually extend over a 2- to 3-hour period which allows for only small quantities of amino acids to be released and absorbed into the blood at any one time. Once in the blood, the excess amino acids are absorbed by cells throughout the entire body within 5 to 10 minutes. Even then, before the effects of the increase in dietary proteins could be reflected in the tears, additional time would be required for the tear producing glands to reassemble these amino acids into proteins and secrete them into the tear film.

When the hourly mean values for the entire population were plotted, the variability displayed by each individual tended to cancel out, giving a relatively flat population curve. The flat nature of this curve without three peaks corresponding to daily meal intake again points to the likelihood that dietary intake has little or no effect on the concentration of proteins in the tear film. Perhaps, if the times of meal intake and dietary content were strictly regulated, the effects of diet on tear protein concentration might be more accurately evaluated. Working with a large number of human subjects often makes such
The only common feature noted among the protein profiles of several subjects was a relatively high total protein concentration measured in the first samples collected each day. It was noted that tear samples collected by these subjects were taken immediately upon awakening. Subjects who did not collect their first tear sample until some time after awakening did not show this peak. This finding suggests that total tear protein concentration becomes elevated during sleep. This might result either from an increased rate of secretion or a decreased rate of elimination of tear proteins in the closed-eye environment.

In previous studies of human tear proteins, differences in protein levels were noted to occur on the basis of sex and age. Females and younger individuals were noted to have higher protein concentration levels, while males and older individuals were found to have lower total tear protein concentration levels. In this study, a population of 11 subjects, 8 male and 3 female, ranging in age from 11 to 59 years (mean 25 years) were studied. Due to the imbalance in number between males and females, as well as the limited number of young and old individuals, valid comparison of protein concentrations in terms of sex or age was not possible. A larger and more evenly balanced population would be required to confirm or reject these earlier findings.

In this investigation, a Beckman Microsome Electrophoresis system using cellulose acetate membranes was employed to separate total protein into three fractional components. This technique, commonly used to evaluate serum proteins, has been previously employed by Hill and others for examining protein fractions in the tear film. By its
design, this system offers several advantages over filter-paper and agar electrophoretic procedures, making it quite applicable for tear studies. The small sample volume required, 2 microliters, permits the collection and evaluation of basic, non-reflexly induced tears. The inert nature of the cellulose membrane with its uniform pore diameter allows for a more rapid and more clearly defined separation of fractions into zones, eliminating the trailing-off of one fraction into another, a problem frequently encountered with other electrophoretic procedures.

By analyzing tear samples collected hourly by each subject, it became possible to monitor the proportional amount of each protein band throughout the day. In this investigation, it was noted that the proportion of these three fractions does not remain constant, but, rather, undergoes considerable variation throughout the day. Within each of the three separable fractions, one or more tear protein components are present. The proportional changes seen for each fraction should be reflective of changes in concentration of the individual tear protein components.

The mode of entry of these protein components into the tear film is uncertain. While some components are believed to be filtrates of blood serum, many are considered to be true glandular secretions. The fact that most of these components display electrophoretic mobilities which differ from those displayed by their serum counterparts suggests their secreted nature. In addition, the absence of some components (lysozyme and tear albumin) from the tears of patients with surgically removed lacrimal glands, again suggests the glandular origin of certain tear proteins. On the basis of molecular size alone, it is
unlikely that tear proteins can readily filter through capillary membranes to form tear fluid components.

Although many tear proteins may differ from serum, thus suggesting them as being true glandular secretions, only limited information regarding their glandular origin and regulation is available.

In 1958, McEwen and Kimura\textsuperscript{66} investigated the glandular source of several secreted protein components and reported them to be derived from one or more of the following sources: the main lacrimal gland, the accessory lacrimal gland, and the conjunctival goblet cells. Lysozyme was noted to originate solely from the lacrimal gland. Its absence in eyes where the lacrimal gland was surgically removed supports this concept. The two types of albumin present in tears were determined to have individual origins: serum albumin originating from conjunctival goblet cells and specific tear albumin arising from the lacrimal gland proper. The globulin fraction with its various subcomponents was less defined and was assumed to be derived from the main lacrimal gland, the accessory lacrimal gland, and the goblet cells. More recently, studies of tear immunoglobulins suggest the lymphatic system (lymph nodes in the conjunctiva) as the source of many of the gamma globulin fractions. That the levels of these components in the tear film do not remain static, but, rather, undergo variation brings up the important question of how the tear producing glands are regulated.

In this investigation, an attempt was made to determine if changes in one fractional component could be correlated with predictable changes in one or both of the other two fractions. It was hoped that if such correlations exist, some insight into the mechanism by which the levels
of these fractional tear components are regulated might be gained.

The findings of this study suggest that, although some components appeared to vary together during the day, no reliable correlations exist for the entire population. The presence of a narrow-range, finely-tuned control mechanism appears to be lacking.

The fact that the proportional amount of each fraction can vary so greatly and still provide the eye with the essentials for maintaining a healthy physiological state is truly remarkable. Previous investigations have shown that when the proportional ratio of two of these fractions (fraction III/I, albumin/lysozyme ratio) becomes significantly out of balance (as occurs with smog eye irritation or industrial gas exposure, whereby the lysozyme fraction is significantly reduced), the eye showed signs of distress in the form of redness and irritation. It was, however, only at extreme levels (III/I ratio) greater than 3.0 (normal, \( \bar{X} = 1.3 \)) that these signs of distress were noted. In both studies, the investigators suggested that the change in protein levels caused a change in tear pH which then initiated the redness and irritation. Similar signs of ocular distress were noted in keratitis-sicca patients who also displayed a reduced or absent lysozyme component. To date, no direct quantitative investigations have been done on the same tear sample relating tear pH to the ratio of individual protein fractions. In this investigation it can be seen that, even though moderate variations in the proportions of each protein fraction can occur throughout the day, the normal health status of the eye is not compromised. Only when the ratios are greatly altered does the eye appear to show signs of distress.
The mean value of the proportion of fraction I : fraction II : fraction III for this sample population is 21.7 : 43.6 : 34.8. This value is similar to that reported by Brunish of 16.4 : 44.8 : 38.8. It differs, however, from that previously reported by Botelho of 17.9 : 23.9 : 58.9. Although such factors as environmental conditions, age, or sex of the subjects may partially account for the differences noted, the type of tears collected (basic vs. reflex) may be the primary source for the discrepancy. In this investigation, when tears were reflexly induced using mechanical irritation, fraction II was seen to decrease, while fractions I and III increased in concentration yielding a proportional ratio of 24 : 25 : 51. This value more closely approximates the value previously reported by Botelho.

In addition to separating tear proteins into fractions, an attempt was made to determine with which of the three separable fractions changes in total protein concentration is most highly correlated. To do this, the proportional amount of each fraction was multiplied by the amount of total protein present in each tear sample. In doing this, the assumption was made that all the stained material in each band was of a proteinaceous nature and that the staining properties of each protein were identical. The calculated values for each fraction and total protein were then plotted.

Graphical comparison and statistical analysis showed that for seven subjects (B, C, D, E, H, J, and K) changes in fraction II were most highly correlated with changes in total protein concentration. For three subjects (A, F, and I) total protein changes appeared to be most highly correlated with changes in fraction III, while one subject (G)
showed no significant correlations with either fraction II or fraction III. No correlative changes in total protein were seen to occur with changes in fraction I. The concentration of fraction I, the lysozyme fraction, appeared rather constant, displaying little change. Thus, it can be speculated that, in most instances, the normal hourly variations in total protein concentration seen throughout the day or from day-to-day, result primarily from changes in concentration of the components in fraction II (globulins). Again, the mechanism controlling these changes remains unknown.

The effects of hydrophilic lens adaptation on the concentration of tear proteins were also examined for four subjects. In a previous investigation, Callendar and Morrison\textsuperscript{11} noted that the decrease in tear protein concentration associated with reflex tearing during adaptation to conventional PMMA lenses provided a useful indicator of the progress of adaptation to lens wear. In that study, a maximum mean decrease in total tear protein of 42 percent was noted, which was associated with corneal edema presumably due to the change in osmotic balance. After adaptation was completed, the protein levels approached their pre-adaptation levels and corneal swelling subsided.

In the present investigation, the mean decrease in total protein concentration was found to be much less, ranging from 2 to 10 percent for the subjects studied. The largest decrease usually occurred during the first hour of lens wear each day. With increased wearing time, the levels increased, approaching their pre-adaptation values. Thus, the hydrophilic lens appears to present less of a stimulus for reflex tearing. Even though a maximum decrease in total protein of 10 percent
was seen, this did not appear to differ significantly from the pre-adaptation level and no detectable signs of edema were noted.

Many of the previous studies of tear protein used reflexly induced tears for analysis. Attempts were made in this investigation to avoid reflexly inducing tear flow in order that basic tears be evaluated. Although the primary goal of this investigation was to determine the mean concentration of total protein in basic tears, attempts were made to compare the differences between the two types of tears using the same technique and instrumentation. To do this, basic and reflexly induced tears were collected from one subject and evaluated. During periods of mechanical irritation, reflexly induced tears, in addition to having a shift toward a lower total protein concentration, also displayed a shift in the proportional amounts of each of the three fractional subcomponents. While the basic tear profile for the particular individual studies reflected a mean ratio of 21.17 : 30.17 : 48.6 for the three separable fractions, when the eye was harshly irritated by mechanical stimulus, this ratio changed to 24 : 25 : 51.

In these reflexly induced tears, total protein showed a mean decrease of 23 percent, while fraction II showed a mean decrease of 17 percent. Both fraction I and fraction III showed a mean increase of 15 percent and 17 percent, respectively, over their basic levels. The reason for their increase may be attributed to the larger input by the main lacrimal gland called into play during reflex stimulation. As previously noted, both lysozyme and tear albumin fractions are believed to originate from secreting cells within the lacrimal gland proper and, as such, may be seen to increase in concentration during
reflex tearing. It should be noted, however, that part of the increase in albumin may be due to an elevated level of serum albumin released from conjunctival goblet cells in response to mechanical irritation. Although the role of increased tear albumin in the defense of the eye is unknown, the increased concentration of lysozyme (with its bactericidal properties) in reflexly induced tears may be part of the eye's natural defense when it becomes challenged. To more fully explore differences between basic and reflex tears, a large sample populating using various forms and types of lacrimating agents would be required.

The primary objectives of this investigation included determining the mean concentration and diurnal changes of both total protein and individual protein fractions in basic tears. The micro-sampling techniques applied have permitted these objectives to be met. From this study, it becomes apparent that tear protein levels do not remain constant throughout the day or from day-to-day. Rather, they undergo fluctuation in what appears to be an unpredictable manner. Because of this variability, little reliable information regarding an individual's tear protein profile can be determined by analyzing only one sample (as has been previously done). Reliable information is obtained only after several samplings are taken and compared.

The electrophoresis technique employed in this investigation has been available for several years. The Abbott Bichromatic Analyzer is of more recent design and is just now becoming routinely used in many hospital laboratories. Its increasing availability in the near future should provide the eye care practitioner with a useful tool for tear
protein evaluations.

Established in this investigation are baseline levels which can be expected within a normal population. Such baselines should prove useful in future tear studies. As more is learned regarding expected changes occurring in protein concentration during disease states and various forms of drug therapy, tear protein concentration evaluation may become an increasingly valuable diagnostic tool.
SUMMARY

1. For the sample population the mean concentration was determined to be 0.81 gm/100 ml (σ = 0.25 gm/100 ml). The range in mean total protein determination ranged from 0.56 gm/100 ml (σ = 0.11 gm/100 gm) to 1.11 gm/100 ml (σ = 0.46 gm/100 ml). The range in individual protein sample concentrations ran from 0.41 gm/100 ml to 2.00 gm/100 ml.

2. The concentration of total protein in the tears does not remain constant, but varies throughout the day and from day to day. No predictable pattern could be detected.

3. The mean concentration for Fractions I, II, III for the entire population was measured to be 0.19 gm/100 ml (σ = 0.06), 0.39 gm/100 ml (σ = 0.19), and 0.31 gm/100 ml (σ = 0.12), respectively. A large amount of variability was noted among the individual subjects.

4. No predictable diurnal patterns in the changes of these three fractions could be detected. Changes in Fraction II appeared most highly correlated with changes in total protein concentration.

5. The concentration of total protein in the tears decreases when the eye adapts to hydrophilic lenses; however, the effects are less dramatic than those occurring during hard lens adaptation. When the eye is more severely mechanically irritated, the concentration of total protein is reduced markedly and the relative proportions of individual protein fractions becomes altered.
APPENDIX A
SUBJECT PROFILE

Subject "A", a senior optometry student, male, age 24, has had no serious ocular or systemic diseases, Break-up time O.D. 23 sec., O.S. 25 sec.

Spectacle Rx
0.D. -0.50 -1.00 x 167
0.S. -0.25 -0.75 x 010

Keratometer Readings
O.D. 44.75 @ 180; 45.62 @ 90
O.S. 44.62 @ 002; 45.87 @ 92

Subject "B", an office worker, male, age 59, has had no serious ocular diseases. Has a history of mild cardiac disorder.

Break-up time O.D. 18 sec., O.S. 20 sec.

Spectacle Rx
0.D. +0.50 -0.25 x 100
0.S. +0.50 DS

Keratometer Readings
O.D. 43.00 @ 180; 43.00 @ 90
O.S. 43.00 @ 180; 43.00 @ 90

Subject "C", a senior optometry student, female, age 22, has had no serious ocular or systemic disorders. Break-up time: O.D. 18 sec., O.S. 20 sec. Adapted to Bausch and Lomb SoflensesR during this study. O.D. -2.75 U3, O.S. -3.25 U3.

Spectacle Rx
0.D. -3.00 DS
0.S. -3.00 -0.50 x 120

Keratometer Readings
O.D. 43.00 @ 020; 43.25 @ 110
O.S. 43.12 @ 014; 43.00 @ 104

Subject "D", a junior optometry student, male, age 25, has had no serious ocular or systemic diseases. Break-up time: O.D. 28 sec., O.S. 30 sec. Adapted to Bausch and Lomb SoflensesR during this study. O.D. -0.75 J, O.S. -1.50 J.
Subject "E", a university student, male, age 25, has had no serious ocular or systemic diseases. Break-up time: O.D. 28 sec., O.S. 26 sec.

Subject "F", a junior optometry student, male, age 22, has had no serious ocular or systemic diseases. Break-up time: O.D. 18 sec., O.S. 16 sec.

Subject "G", a university graduate student, female, age 34, has had no serious ocular or systemic diseases. Break-up time: O.D. 13 sec., O.S. 17 sec. Adapted to Bausch and Lomb Soflenses during this study. O.D. -3.00 D, O.S. -3.00 D.

Subject "H", a junior optometry student, male, age 21, has had no serious ocular or systemic diseases. Break-up time: O.D. 23 sec., O.S. 19 sec.
Subject "I", a high school junior, male, age 16, has had no serious ocular or systemic diseases. Break-up time: O.D. 21 sec., O.S. 24 sec.

Spectacle Rx | Keratometer readings
---|---
0.0 D. -2.25 DS | O.D. 45.75 @ 180; 46.25 @ 090
0.0 S. -2.25 DS | O.S. 46.25 @ 180; 46.25 @ 090

Subject "J", a junior optometry student, male, age 24, has had no serious ocular or systemic diseases. Break-up time: O.D. 21 sec., O.S. 24 sec.

Spectacle Rx | Keratometer readings
---|---
0.0 D. -0.75 -0.75 x 160 | O.D. 44.00 @ 180; 45.00 @ 090
0.0 S. -0.75 -0.50 x 020 | O.S. 44.00 @ 014; 45.00 @ 104

Subject "K", a university office worker, female, age 29, has had no serious ocular or systemic disorders. Break-up time: O.D. 18 sec., O.S. 16 sec. Adapted to Bausch and Lomb Soflenses® during this study. O.D. -2.75 N, O.S. -2.50 N.

Spectacle Rx | Keratometer readings
---|---
0.0 D. -2.50 DS | O.D. 44.75 @ 180; 45.00 @ 090
0.0 S. -2.50 -0.50 x 003 | O.S. 44.75 @ 180; 45.12 @ 090

Note: Break-up times for each eye reflect the average of three determinations.
Traditional photometric measurements involve using a photometer and measuring the absorbance of some unknown solution against the absorbance of a reference solution. A sample of unknown concentration was introduced into a reagent solution causing a chromogen (color carrier) in the solution to be developed or reduced as a result of a chemical reaction. This change in chromogen concentration produced distinct and measurable changes in the amount of light absorbed. The absorbance measured by the photometer was shown to be proportional to the concentration of the chromogen, which in return, was a function of the concentration of the unknown.

Limitations of this form of photometry result primarily from variations between the state of the reference solution and the sample solution. Such variations may include debris on the container cell windows, particles or turbidity in the solutions, optical path differences, or detector sensitivity variations.

Bichromatic photometry eliminates or minimizes many of these variables by measuring differences in absorbance between a peak and side band wavelength for each cell. Since both wavelengths are passed alternately through a single optical system in each cell, both will be affected equally by variations unique to each cell. Any variations that may exist would add a constant (k) amount of absorbance to each
wavelength, thus making the difference the same.

**MATHEMATICAL PRINCIPLES**

The absorption difference (\(A_d\)) is related to the concentrations of the chromogen and the unknown as follows:

\[
A_d = \log \frac{l_s}{l_p} = \log l_s - \log l_p \quad (1)
\]

\[
C_c = K_1 A_d \quad (2)
\]

\[
C_u = K_1 K_2 A_d \quad (3)
\]

Where:

- \(A_d\) = absorbance difference
- \(l_p\) = intensity of light transmitted at peak wavelength
- \(l_s\) = intensity of light transmitted at sideband wavelength
- \(C_c\) = concentration of chromogen
- \(K_1\) = a constant which is a function of the thickness of the solution and the wavelength of the light being utilized
- \(C_u\) = concentration of the unknown sample
- \(K_2\) = a constant that relates the concentration of the unknown with the temperature at which the reaction occurs and the dilution of the fixed sample.

The absorption difference is also related to the Beer-Lambert laws. Absorbance (\(A\)), as measured with a photometer, is defined as the log of the ratio of the intensity of light incident on a solution (\(l_i\)) to the intensity of light transmitted by the solution \(l_t\):

\[
A = \log \frac{l_i}{l_t} = \log l_i - \log l_t \quad (4)
\]

Lambert's Law states that the amount of light absorbed is proportional to the thickness of the solution (\(b\)) in centimeters, multiplied by the absorption coefficient (\(a\)).

\[
A = ba \quad (5)
\]
The absorption coefficient \( a \) is the fraction by which the initial incident light of a particular wavelength is reduced per centimeter of solution thickness.

Beer's Law states that the amount of light absorbed is a linear function of the concentration \( c \) of the solution and optical path. Therefore, the absorption coefficient \( a \) previously described is a function of solution concentration:

\[
a = ac \quad (6)
\]

\( a \) = molar extinction coefficient of the solution at a particular wavelength

\( c \) = the concentration of solution in moles per liter

Combining Beer's and Lambert's laws, we find the expression for the attenuation of a single wavelength of light in an absorbing solution to be:

\[
A = abc = \log I_i - \log I_t \quad (7)
\]

Since \( a \), the molar extinction coefficient, and \( b \), the optical pathlength, are constant for a particular absorbing material, the concentration \( c \) of a solution is directly proportional to absorbance:

\[
c = \log I_i - \log I_t \quad (8)
\]

In bichromatic photometry, the absorption difference \( A_d \) between the peak wavelength \( \lambda_p \) and the side wavelength \( \lambda_s \) is used in determining the concentration of an unknown solution.

\[
A_d = \lambda_p - \lambda_s \quad (9)
\]

Applying Lambert's and Beer's laws, the difference in absorption can be defined as:

\[
A_d = (a_p - a_s)bc - \log I_{ts} - \log I_{tp} \quad (10)
\]
where:

\[ a_p = \text{molar extinction coefficient for the solution at peak wavelength} \]

\[ a_s = \text{molar extinction coefficient for the solution at a side band wavelength} \]

\[ b = \text{thickness of the solution} \]

\[ c = \text{concentration in moles per liter} \]

Since \( a_p, a_s, \) and \( b \) are constant, this equation reduces to:

\[ A_d = c = \log l_{ts} - \log l_{tp} \quad (11) \]

Therefore, it can be concluded that \( A_d \) is linearly related to chromogen concentration and is a function of the difference between the molar absorptivities of the chromogen at two wavelengths. Thus, \( A_d \) changes when the chromogen concentration changes.
APPENDIX C

CHEMICAL AGENTS

ELECTROPHORESIS

The following chemical agents were purchased and used as identification markers for electrophoresis:

HUMAN GAMMA GLOBULIN

Miles Laboratories, Inc., Elkhart, Indiana

Lot No: 42

Contents:  
- Ash sulfated: 0.30%
- Chloride: 0.6 mg/gm
- Moisture: 1.0%
  Karl Fischer

Protein: 97.0%
  By Nitrogen Analyzer
  Dry Weight Basis
  Protein Factor 6.25

pH (6% Solution): 5.9

Electrophoresis: Cellulose acetate; Barbital Buffer pH 8.6, 0.075 ionic strength

Purity: Gamma Globulins 99% (Fraction II)

HUMAN ALPHA GLOBULINS IV

Miles Laboratories, Inc., Elkhart, Indiana

Lot No: 28

Contents:  
- Ash Sulfated: 7.3%
- Chloride: 47.6 mg/gm
- Moisture: 3.7%
  Karl Fischer
Protein 80.0%  
By Nitrogen Analyzer
Dry Weight Basis
Protein Factor 6.25

pH (7% Solution) 6.9

Electrophoresis: Cellulose acetate; Barbitol Buffer, pH 8.6, 0.075 ionic strength

HUMAN ALBUMIN

Miles Laboratories, Inc., Elkhart, Ind.
Lot No: 36
Contents: Ash Sulfated 0.8%
Chloride 2.5 mg/gm
Moisture 2.2%
Karl Fischer
Protein 98.0%
By Nitrogen Analyzer
Dry Weight Basis
Protein Factor 6.22

pH (7% Solution) 5.0

Electrophoresis: Cellulose acetate; Barbitol Buffer, pH 8.6, 0.075 ionic strength

Purity: Albumin 98%
(Fraction V)

LYSOZYME, GRADE 1: FROM EGG WHITE

Sigma Chemical Company, St. Louis, Mo.
Lot No: 57C-8025
Characteristics: 3X crystallized
dialyzed and lyophilized powder activity:
38,500 units/mg solid
protein content: 90%
ABA-SPECTROPHOTOMETER

This instrument was calibrated and checked for linearity using solutions formulated from Total Protein Standard®.

HUMAN PROTEIN STANDARD

Dade Division, Scientific Products, Columbus, Ohio
Lot No: PRS-425
Characteristics: lyophilized product prepared from crystallized albumin
Total Protein (albumin) - calculated at 6.25 x total nitrogen...8.2g/dl
Product stability - reconstituted 5 days at 2-8°C.
Vial-to-vial consistency - maximum variation of ±1% by weight.
Moisture content - less than 1% by weight.
Reliability of assigned value - The assigned value for total nitrogen is confirmed by replicate analyses by several analysts using well-established methods.
Membrane filtered and low bacterial count.

A-GEN TOTAL PROTEIN REAGENT

Abbott Laboratories, Diagnostic Division, Pasadena, Ca.
List No: 6027-02
Contents:

<table>
<thead>
<tr>
<th>Component</th>
<th>mmole/liter of Reaction Mixture</th>
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</thead>
<tbody>
<tr>
<td>Copper Tartrate</td>
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<tr>
<td>Lithium Hydroxide</td>
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</tr>
<tr>
<td>Sodium Tartrate</td>
<td>9.1</td>
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</table>

Inert fillers are present but have no effect on the measurement of protein.
Note:

Care was taken to avoid using any of chemical agents beyond their shelf-life expiration date.
APPENDIX D

PRECISION AND ACCURACY

In this investigation, protein concentrations were determined using a biuret reaction. This method of protein analysis is considered to be one of the most accurate available. The accuracy and reliability of measurements made in this investigation are further enhanced by the use of the bichromatic photometer system incorporated in the Abbott ABA-50. This system offers many advantages over the traditional monochromatic photometry (see Appendix B).

Precision

Precision is a measure of the consistency of a measurement. The standard deviation of a statistic is one of the most useful indicators of precision.

Maximizing the precision of the method employed in this investigation involves accounting for and controlling numerous possible sources of error. Several potential sources of error resulting from instrumentation or procedure and their controls or solutions are as follows:

<table>
<thead>
<tr>
<th>FACTOR</th>
<th>PROBLEM</th>
<th>SOLUTION OR CONTROL</th>
</tr>
</thead>
<tbody>
<tr>
<td>sample size</td>
<td>change in sample size gives more endpoint product and a different apparent concentration</td>
<td>1. use constant amount for samples</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. carefully check for air bubbles, etc. in the micropipettes and sample capillary tubes</td>
</tr>
<tr>
<td>FACTOR</td>
<td>PROBLEM</td>
<td>SOLUTION OR CONTROL</td>
</tr>
<tr>
<td>---------------------</td>
<td>-------------------------------------------------------------------------</td>
<td>-----------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>temperature</td>
<td>temperature variations cause changes in reaction rate and the endpoint may occur at a different point</td>
<td>1. calibrate before each use for proper temperature</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. avoid room drafts</td>
</tr>
<tr>
<td>linearity</td>
<td>nonlinearity gives wrong value</td>
<td>1. check linearityity with standard solutions</td>
</tr>
<tr>
<td>cuvette imperfections</td>
<td>imperfections in cuvette may give inconsistent readings due to differential absorption</td>
<td>1. use new cuvettes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. visually inspect each before use</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3. evaluate absorption of each cell using Abbott ABA-50 prior to use</td>
</tr>
<tr>
<td>incubation</td>
<td>variations in incubation time gives possible error if reaction rate is slightly different</td>
<td>1. check all samples at 10 minutes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. keep incubation interval constant for all sampling</td>
</tr>
<tr>
<td>sample evaporation</td>
<td>concentrated tears because of evaporation</td>
<td>1. tears are sealed immediately after collected</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. samples remain sealed until analysis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3. ends are filed off and tears are carefully collected into micro-pipettes, then dispensed into the sample chamber</td>
</tr>
<tr>
<td>substrate reflections</td>
<td>added light of a particular wavelength may give false reading in absorption</td>
<td>1. keep from direct light</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. keep ambient lighting conditions constant</td>
</tr>
<tr>
<td>change in baseline</td>
<td>small change in current or temperature produce resultant changes in baseline giving falsely high or low values</td>
<td>1. record baseline relative to zero of each cell with all stable</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. record baseline level at zeroing and calibration</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3. check before each set of eight readings that the baseline remains zero and rezero if changed</td>
</tr>
</tbody>
</table>
The linearity of the Abbott ABA-50 for measuring total protein concentration was established using control solutions of known concentration. The data from seven trials conformed to a linear regression line of $y = 0.03 + 0.99x$ with an $r^2$ value of 1.00 (Figure 11).

The precision of the instrument was examined by measuring the concentration of thirty samples of control solution of known concentration (4.10 G/dL) (see Table 5). The mean level measured was 4.10 G/dL with a standard deviation ($\sigma = 0.03$ G/dL). This shows that, with good technique, this procedure for protein determination can produce highly consistent measurements.

**ACCURACY**

Accuracy is defined as the agreement of the instrument readings with the true or known values.

**Chemical Basis for Accuracy**

Biuret is a compound having the following structure:

$$\text{CONH}_2 \quad \text{NH} \quad \text{CONH}_2$$

When biuret is treated with an alkaline potassium copper tartrate
solution, two biuret molecules are joined to form a violet colored complex of the following structure:

\[
\begin{array}{c}
\text{CONH}_2 - \text{Cu} - \text{NH}_2\text{CO} \\
\text{NH} \\
\text{OH} \\
\text{OCH}_2 - \text{K} - \text{K-NH}_2\text{CO}
\end{array}
\]

The violet color produced is due to the formation of a coordination complex between cupric ion and protein nitrogen in an alkaline medium. The amount of violet color produced is a measure of the quantity of biuret present, which, in turn, is in direct proportion to the protein concentration.

The biuret reaction is given by all compounds having two amide or peptide bonds linked either directly or through an intermediary carbon atom. The color depends upon the presence of two or more peptide linkages of the following type:

\[
\begin{array}{c}
-C-C-N-C-
\end{array}
\]

Tripeptides and proteins possess the following structure and, as such, yield a colored endproduct:

\[
\begin{array}{c}
\text{O} \\
-\text{C-C-NH-C-C-NH-C-}
\end{array}
\]

Since two peptide bonds are required for the biuret reaction to occur, dipeptides and amino acids do not give this reaction.

In this investigation, Total Protein A-Gent\textsuperscript{R} is used as the substrate for the reaction to occur. This substance, containing the
biuret compound, has been formulated to optimize color development, reaction sensitivity, and shelf-life stability.
APPENDIX E

STATISTICAL FUNCTIONS

Moments of Central Tendency

\[ m_2 = \frac{1}{n} \sum x_i^2 - \bar{x}^2 \]  

(12)

\[ m_3 = \frac{1}{n} \sum x_i^3 - 3/\bar{x} \sum x_i^2 + 2\bar{x}^3 \]  

(13)

\[ m_4 = \frac{1}{n} \sum x_i^4 - 4/\bar{x} \sum x_i^3 + 6/\bar{x}^2 \sum x_i^2 - 3\bar{x}^4 \]  

(14)

Coefficient of Skewness \( \gamma_1 \)

Coefficient of Kurtosis \( \gamma_2 \)

\[ \gamma_1 = \left( \frac{m_3}{m_2} \right)^{3/2} \]  

(15)

\[ \gamma_2 = \left( \frac{m_4}{m_2} \right)^2 \]  

(16)
Cyclic Function Determination

Let \( x_1 = \sin \frac{2\pi t}{\text{period}} \) \hfill (17)

\( x_2 = \cos \frac{2\pi t}{\text{period}} \) \hfill (18)

\[ y = \alpha + (\beta \cos K)x_1 + (\beta \sin K)x_2 \] \hfill (19)

Fit multiple regression and find

\[ \hat{y} = \hat{\alpha} + \hat{\gamma}_1 x_1 + \hat{\gamma}_2 x_2 \]; now we use \( \hat{\gamma}_1, \hat{\gamma}_2 \) to estimate both \( \beta \) and \( K \):

\[ \hat{\gamma}_1 = \sqrt{\hat{\gamma}_1^2 + \hat{\gamma}_2^2} \]

\[ \hat{\gamma}_2 = \frac{\hat{\gamma}_1}{\sqrt{\hat{\gamma}_1^2 + \hat{\gamma}_2^2}} \] \hfill (21)

\[ \beta = \hat{\gamma}_1^2 + \hat{\gamma}_2^2 \] \hfill (22)

\[ \cos K = \frac{\hat{\gamma}_1}{\sqrt{\hat{\gamma}_1^2 + \hat{\gamma}_2^2}} \] \hfill (23)

\[ \sin K = \frac{\hat{\gamma}_2}{\sqrt{\hat{\gamma}_1^2 + \hat{\gamma}_2^2}} \] \hfill (24)

\[ y = \text{intercept } \beta_1 \left( \sin \frac{2\pi t}{p} \right) + \beta_2 \left( \cos \frac{2\pi t}{p} \right) \] \hfill (25)
### Significance Levels for Binomial Statistical Procedure

<table>
<thead>
<tr>
<th>Sample Size</th>
<th>Alpha</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>0.031</td>
<td>( X = 6 ) or ( X = 0 )</td>
</tr>
<tr>
<td>9</td>
<td>0.039</td>
<td>( X \geq 8 ) or ( X \leq 1 )</td>
</tr>
<tr>
<td>10</td>
<td>0.109</td>
<td>( X \geq 9 ) or ( X \leq 1 )</td>
</tr>
<tr>
<td>11</td>
<td>0.065</td>
<td>( X \geq 9 ) or ( X \leq 2 )</td>
</tr>
<tr>
<td>12</td>
<td>0.038</td>
<td>( X \geq 10 ) or ( X \leq 2 )</td>
</tr>
<tr>
<td>13</td>
<td>0.092</td>
<td>( X \geq 10 ) or ( X \leq 3 )</td>
</tr>
<tr>
<td>14</td>
<td>0.056</td>
<td>( X \geq 11 ) or ( X \leq 3 )</td>
</tr>
</tbody>
</table>
REFERENCES


15. Fleming, A., On a remarkable bacteriolytic substance found in 


17. Casalli, F., Schumacher, H., Uber den Nachweis des Tränenlysozym 
   1954.

   tears, part I: Lysozyme and its correlation with Keratoconjunctivitis 

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    electrophoresis of tears, part 3: Human tears and their high 

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62. Thayer, G.R., Measurement of Calcium in Human Tears: Levels and Physiological Responses, A Thesis, The Ohio State University, Columbus, Ohio, 1974


65. Hathaway, R.A., The Compatibility of a Hydrophilic Lens Polymer (HEMA) with the Human Tear Fluid, A Thesis, The Ohio State University, Columbus, Ohio, 1976


<table>
<thead>
<tr>
<th>Year</th>
<th>Investigator</th>
<th>Technique</th>
<th>Total Protein gr/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1882</td>
<td>Møgaard²</td>
<td>Nitric Acid Precipitation</td>
<td>1.4638</td>
</tr>
<tr>
<td>1922</td>
<td>v. Rotth⁴</td>
<td>Refractometry</td>
<td>0.25 to 0.6</td>
</tr>
<tr>
<td>1930</td>
<td>Ridley⁵</td>
<td>Salting-out</td>
<td>0.669</td>
</tr>
<tr>
<td>1952</td>
<td>Junnola⁶</td>
<td>Colorimetric Nephelometry</td>
<td>0.136 to 0.592</td>
</tr>
<tr>
<td>1953</td>
<td>Salik⁷</td>
<td>Colorimetric Polography</td>
<td>4.52</td>
</tr>
<tr>
<td>1957</td>
<td>Brunish⁸</td>
<td>Filter Paper Electrophoresis and Spectrophotometer</td>
<td>0.4 to 0.6</td>
</tr>
<tr>
<td>1958</td>
<td>Erickson⁹</td>
<td>Filter Paper Electrophoresis in Lysozyme Equivalents</td>
<td>0.39 to 2.9</td>
</tr>
<tr>
<td>1973</td>
<td>Tapasztó¹⁰</td>
<td>Spectrocolorimeter</td>
<td>0.6 to 0.8</td>
</tr>
<tr>
<td>1974</td>
<td>Callendar¹¹</td>
<td>Lowry Technique</td>
<td>1.02</td>
</tr>
<tr>
<td>1975</td>
<td>Bluestone²⁴</td>
<td>Lowry Technique</td>
<td>0.1 to 2.7</td>
</tr>
<tr>
<td>Year</td>
<td>Investigators</td>
<td>IgA</td>
<td>IgD</td>
</tr>
<tr>
<td>--------</td>
<td>--------------------------------------</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>1963</td>
<td>Chodirker &amp; Tomasi27</td>
<td>7</td>
<td>---</td>
</tr>
<tr>
<td>1968</td>
<td>Bracciolini28</td>
<td>24</td>
<td>---</td>
</tr>
<tr>
<td>1968</td>
<td>Barnett29</td>
<td>31</td>
<td>---</td>
</tr>
<tr>
<td>1969</td>
<td>Little, Centifanto, Kauffman30</td>
<td>21</td>
<td>---</td>
</tr>
<tr>
<td>1970</td>
<td>Bazzi, Cattaneo, Migone, and Farina31</td>
<td>23</td>
<td>---</td>
</tr>
<tr>
<td>1971</td>
<td>Brauninger &amp; Centifanto32</td>
<td>9-50</td>
<td>---</td>
</tr>
<tr>
<td>1973</td>
<td>McClellan, Whitney, Newman, Alansmith13</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>1975</td>
<td>Sen, Sarin, Mani, Saha33</td>
<td>24.6</td>
<td>0</td>
</tr>
<tr>
<td>1975</td>
<td>Bluestone, Easty, Goldberg, Jones, Pettit</td>
<td>107*</td>
<td>0</td>
</tr>
<tr>
<td>1978</td>
<td>Sen, Sarin, Mathur, and Saha</td>
<td>30.7*</td>
<td>---</td>
</tr>
</tbody>
</table>

Trace = less than 1 mg/100 ml; --- Not studied; * ug/mg protein; ** ng/ml.
<table>
<thead>
<tr>
<th>Subject</th>
<th>N</th>
<th>Mean</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>75</td>
<td>0.56</td>
<td>0.11</td>
</tr>
<tr>
<td>B</td>
<td>68</td>
<td>0.68</td>
<td>0.12</td>
</tr>
<tr>
<td>C</td>
<td>55</td>
<td>0.74</td>
<td>0.12</td>
</tr>
<tr>
<td>D</td>
<td>62</td>
<td>0.77</td>
<td>0.13</td>
</tr>
<tr>
<td>E</td>
<td>70</td>
<td>0.78</td>
<td>0.20</td>
</tr>
<tr>
<td>F</td>
<td>75</td>
<td>0.78</td>
<td>0.20</td>
</tr>
<tr>
<td>G</td>
<td>64</td>
<td>0.83</td>
<td>0.18</td>
</tr>
<tr>
<td>H</td>
<td>70</td>
<td>0.87</td>
<td>0.17</td>
</tr>
<tr>
<td>I</td>
<td>64</td>
<td>0.87</td>
<td>0.21</td>
</tr>
<tr>
<td>J</td>
<td>69</td>
<td>0.92</td>
<td>0.19</td>
</tr>
<tr>
<td>K</td>
<td>65</td>
<td>1.11</td>
<td>0.46</td>
</tr>
</tbody>
</table>
### TABLE 4

**POPULATION FREQUENCIES OF TOTAL PROTEIN CONCENTRATION**

<table>
<thead>
<tr>
<th>Range gm/100 ml</th>
<th>Number</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 0.19</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.2 - 0.39</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.4 - 0.59</td>
<td>101</td>
<td>13.8%</td>
</tr>
<tr>
<td>0.6 - 0.79</td>
<td>314</td>
<td>42.9%</td>
</tr>
<tr>
<td>0.8 - 0.99</td>
<td>200</td>
<td>27.32%</td>
</tr>
<tr>
<td>1.00 - 1.19</td>
<td>63</td>
<td>8.61%</td>
</tr>
<tr>
<td>1.20 - 1.39</td>
<td>32</td>
<td>4.37%</td>
</tr>
<tr>
<td>1.40 - 1.59</td>
<td>10</td>
<td>1.37%</td>
</tr>
<tr>
<td>1.60 - 1.79</td>
<td>2</td>
<td>0.27%</td>
</tr>
<tr>
<td>1.80 - 1.99</td>
<td>9</td>
<td>1.23%</td>
</tr>
</tbody>
</table>

N = 732

Mean = 0.81 gm/100 ml

Standard Deviation = 0.25 gm/100 ml

Median = 0.76 gm/100 ml

Mode = 0.73 gm/100 ml
**TABLE 5**

**PRECISION AND ACCURACY**

Results of thirty trials measuring a 4.10 gram/100 ml. standard solution

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>4.08</td>
<td>4.08</td>
<td>4.09</td>
<td>4.14</td>
<td>4.08</td>
</tr>
<tr>
<td>4.12</td>
<td>4.06</td>
<td>4.11</td>
<td>4.08</td>
<td>4.08</td>
</tr>
<tr>
<td>4.14</td>
<td>4.07</td>
<td>4.07</td>
<td>4.10</td>
<td>4.07</td>
</tr>
<tr>
<td>4.07</td>
<td>4.08</td>
<td>4.08</td>
<td>4.09</td>
<td>4.09</td>
</tr>
<tr>
<td>4.11</td>
<td>4.14</td>
<td>4.10</td>
<td>4.10</td>
<td>4.14</td>
</tr>
<tr>
<td>4.10</td>
<td>4.14</td>
<td>4.14</td>
<td>4.12</td>
<td>4.10</td>
</tr>
</tbody>
</table>

Arithmetic Mean = 4.10 g/100 ml.

Standard Deviation = 0.03

Standard Error = 0.00

Range = 4.06 to 4.14
### TABLE 6

**BINOMIAL DISTRIBUTION THEORY**

<table>
<thead>
<tr>
<th>Fraction 1:</th>
<th>+ + ± - + ± - - + ± + + ±</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction 2:</td>
<td>+ - + + - + + - + - - -</td>
</tr>
<tr>
<td>Fraction 3:</td>
<td>- + - + + + - + - + - +</td>
</tr>
</tbody>
</table>

- $H_0 =$ two fractions vary by chance
- $H_1 =$ two fractions do not vary by chance

For $N = 13$, the significance level for accepting or rejecting the hypothesis is:

$B(13, \frac{1}{2}) \Rightarrow \alpha = (2)(0.461) = 0.0922$, \( \{X \geq 10 \text{ or } X \leq 3\} \)

<table>
<thead>
<tr>
<th>Fractions Compared</th>
<th>Number of Matches</th>
<th>Hypothesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>$X_{1,11}$</td>
<td>4</td>
<td>do not reject</td>
</tr>
<tr>
<td>$X_{11,111}$</td>
<td>2</td>
<td>reject</td>
</tr>
<tr>
<td>$X_{1,111}$</td>
<td>8</td>
<td>do not reject</td>
</tr>
</tbody>
</table>
**TABLE 7**

**CORRELATIVE CHANGES IN TEAR PROTEINS**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Fraction</th>
<th>Details</th>
</tr>
</thead>
</table>
| Subject A | A. Fraction I and Fraction II vary by chance.  
             n = 14  
             α = 0.056 | B. Fraction I and Fraction III vary by chance.  
             C. Fraction II and Fraction III move oppositely. |
| Subject B | A. Fraction I and Fraction II move in opposite directions.  
             n = 12  
             α = 0.038 | B. Fraction I and Fraction III vary by chance.  
             C. Fraction II and Fraction III vary by chance. |
| Subject C | A. Fraction I and Fraction II vary by chance.  
             n = 10  
             α = 0.10 | B. Fraction I and Fraction III vary by chance.  
             C. Fraction II and Fraction III vary oppositely. |
| Subject D | A. Fraction I and Fraction II vary in opposite directions.  
             n = 11  
             α = 0.06 | B. Fraction I and Fraction III vary by chance.  
             C. Fraction II and Fraction III vary by chance. |
| Subject E | A. All Fractions vary by chance.  
             n = 12  
             α = 0.038 | C. |
| Subject F | A. Fraction I and Fraction II vary by chance.  
             n = 13  
             α = 0.02 | B. Fraction I and Fraction III vary by chance.  
             C. Fraction II and Fraction III vary oppositely. |
| Subject G | A. Fraction I and Fraction II vary by chance.  
             n = 6  
             α = 0.03 | B. Fraction I and Fraction III vary by chance.  
             C. Fraction II and Fraction III vary in same direction. |
| Subject H | A. Fraction I and Fraction II vary by chance.  
             n = 12  
             α = 0.02 | B. Fraction I and Fraction III vary oppositely.  
             C. Fraction II and Fraction III vary oppositely. |
| Subject I | A. Fraction I and Fraction II move in opposite directions.  
             n = 14  
             α = 0.05 | B. Fraction I and Fraction III vary by chance.  
             C. Fraction II and Fraction III move in opposite directions. |
| Subject J | A. Fraction I and Fraction II vary by chance.  
             n = 9  
             α = 0.039 | B. Fraction I and Fraction III vary by chance.  
             C. Fraction II and Fraction III vary by chance. |
| Subject K | A. Fraction I and Fraction II go in opposite directions.  
             n = 12  
             α = 0.038 | B. Fraction I and Fraction III vary by chance.  
             C. Fraction II and Fraction III vary by chance. |
# TABLE 8

**MEAN PROPORTION OF EACH FRACTION FOR INDIVIDUAL SUBJECTS AND POPULATION**

<table>
<thead>
<tr>
<th>Subject</th>
<th>N</th>
<th>% Fraction I</th>
<th>% Fraction II</th>
<th>% Fraction III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$\bar{x}$</td>
<td>$\sigma$</td>
<td>$\bar{x}$</td>
</tr>
<tr>
<td>A</td>
<td>15</td>
<td>38  0.03</td>
<td>41  0.04</td>
<td>21  0.04</td>
</tr>
<tr>
<td>B</td>
<td>13</td>
<td>20  0.04</td>
<td>53  0.06</td>
<td>27  0.03</td>
</tr>
<tr>
<td>C</td>
<td>11</td>
<td>19  0.02</td>
<td>40  0.05</td>
<td>39  0.06</td>
</tr>
<tr>
<td>D</td>
<td>14</td>
<td>24  0.05</td>
<td>48  0.09</td>
<td>28  0.06</td>
</tr>
<tr>
<td>E</td>
<td>13</td>
<td>18  0.05</td>
<td>47  0.12</td>
<td>35  0.10</td>
</tr>
<tr>
<td>F</td>
<td>14</td>
<td>16  0.03</td>
<td>34  0.06</td>
<td>50  0.06</td>
</tr>
<tr>
<td>G</td>
<td>14</td>
<td>19  0.05</td>
<td>37  0.08</td>
<td>44  0.04</td>
</tr>
<tr>
<td>H</td>
<td>13</td>
<td>21  0.04</td>
<td>53  0.05</td>
<td>27  0.04</td>
</tr>
<tr>
<td>I</td>
<td>15</td>
<td>19  0.03</td>
<td>39  0.04</td>
<td>43  0.03</td>
</tr>
<tr>
<td>J</td>
<td>11</td>
<td>23  0.05</td>
<td>44  0.07</td>
<td>34  0.04</td>
</tr>
<tr>
<td>K</td>
<td>13</td>
<td>28  0.02</td>
<td>41  0.04</td>
<td>31  0.02</td>
</tr>
<tr>
<td>Population</td>
<td>146</td>
<td>21.70 6.18</td>
<td>43.60 6.54</td>
<td>34.8 9.19</td>
</tr>
<tr>
<td>Subject</td>
<td>N</td>
<td>Fraction 1</td>
<td>Fraction 2</td>
<td>Fraction 3</td>
</tr>
<tr>
<td>---------</td>
<td>---</td>
<td>------------</td>
<td>------------</td>
<td>------------</td>
</tr>
<tr>
<td>A</td>
<td>15</td>
<td>0.25 ± 0.05</td>
<td>0.27 ± 0.06</td>
<td>0.14 ± 0.04</td>
</tr>
<tr>
<td>B</td>
<td>13</td>
<td>0.15 ± 0.04</td>
<td>0.38 ± 0.11</td>
<td>0.19 ± 0.03</td>
</tr>
<tr>
<td>C</td>
<td>11</td>
<td>0.13 ± 0.03</td>
<td>0.29 ± 0.08</td>
<td>0.29 ± 0.09</td>
</tr>
<tr>
<td>D</td>
<td>14</td>
<td>0.23 ± 0.04</td>
<td>0.48 ± 0.18</td>
<td>0.27 ± 0.06</td>
</tr>
<tr>
<td>E</td>
<td>13</td>
<td>0.16 ± 0.03</td>
<td>0.48 ± 0.35</td>
<td>0.33 ± 0.09</td>
</tr>
<tr>
<td>F</td>
<td>14</td>
<td>0.15 ± 0.04</td>
<td>0.32 ± 0.06</td>
<td>0.47 ± 0.12</td>
</tr>
<tr>
<td>G</td>
<td>14</td>
<td>0.16 ± 0.03</td>
<td>0.31 ± 0.11</td>
<td>0.30 ± 0.07</td>
</tr>
<tr>
<td>H</td>
<td>13</td>
<td>0.21 ± 0.06</td>
<td>0.58 ± 0.32</td>
<td>0.28 ± 0.13</td>
</tr>
<tr>
<td>I</td>
<td>15</td>
<td>0.17 ± 0.05</td>
<td>0.35 ± 0.07</td>
<td>0.39 ± 0.08</td>
</tr>
<tr>
<td>J</td>
<td>11</td>
<td>0.25 ± 0.05</td>
<td>0.48 ± 0.12</td>
<td>0.37 ± 0.07</td>
</tr>
<tr>
<td>K</td>
<td>13</td>
<td>0.26 ± 0.09</td>
<td>0.37 ± 0.12</td>
<td>0.28 ± 0.09</td>
</tr>
<tr>
<td>Population</td>
<td>146</td>
<td>0.19 ± 0.06</td>
<td>0.39 ± 0.19</td>
<td>0.31 ± 0.12</td>
</tr>
</tbody>
</table>
TABLE 10

PROTEIN CONCENTRATION IN NORMAL TEARS VS. REFLEX TEARS

<table>
<thead>
<tr>
<th>Time</th>
<th>Normal Total Protein gm/100 ml</th>
<th>Percentage Fraction I</th>
<th>Percentage Fraction II</th>
<th>Percentage Fraction III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>20%</td>
<td>28%</td>
<td>52%</td>
</tr>
<tr>
<td>10:00 am</td>
<td>0.72</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12:00 N</td>
<td>0.64</td>
<td>21%</td>
<td>30%</td>
<td>49%</td>
</tr>
<tr>
<td>2:00 pm</td>
<td>0.60</td>
<td>22%</td>
<td>28%</td>
<td>50%</td>
</tr>
<tr>
<td>4:00 pm</td>
<td>0.67</td>
<td>21%</td>
<td>35%</td>
<td>44%</td>
</tr>
<tr>
<td>6:00 pm</td>
<td>0.65</td>
<td>22%</td>
<td>30%</td>
<td>48%</td>
</tr>
<tr>
<td>8:00 pm</td>
<td>0.61</td>
<td>21%</td>
<td>30%</td>
<td>49%</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>21.17</td>
<td>30.17</td>
<td>48.67</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reflex Total Protein gm/100 ml</th>
<th>Percentage Fraction I</th>
<th>Percentage Fraction II</th>
<th>Percentage Fraction III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>21%</td>
<td>21%</td>
<td>58%</td>
</tr>
<tr>
<td>12:00 N</td>
<td>0.55</td>
<td>25%</td>
<td>23%</td>
</tr>
<tr>
<td>2:00 pm</td>
<td>0.43</td>
<td>25%</td>
<td>20%</td>
</tr>
<tr>
<td>4:00 pm</td>
<td>0.53</td>
<td>27%</td>
<td>26%</td>
</tr>
<tr>
<td>6:00 pm</td>
<td>0.56</td>
<td>22%</td>
<td>34%</td>
</tr>
<tr>
<td>8:00 pm</td>
<td>0.42</td>
<td>24%</td>
<td>26%</td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
<td>25</td>
<td>51</td>
</tr>
</tbody>
</table>

\[
\bar{x} = 0.65, \sigma = 0.4 \\
\bar{x} = 21.17, \sigma = 0.75 \\
\bar{x} = 30.17, \sigma = 2.56 \\
\bar{x} = 48.67, \sigma = 2.66 \\
\bar{x} = 0.50, \sigma = 0.06 \\
\bar{x} = 24, \sigma = 2.19 \\
\bar{x} = 51, \sigma = 5.06 \\
\bar{x} = 5.14
\]
TABLE II

CORRELATIVE CHANGES IN TOTAL PROTEIN AND INDIVIDUAL PROTEIN FRACTIONS

FRIEDMAN RANKING TEST

<table>
<thead>
<tr>
<th>SUBJECT</th>
<th>SUM OF RANKINGS</th>
<th>DIFFERENCES BETWEEN FRACTIONS (α = 0.10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ΣR₁</td>
<td>ΣR₁¹</td>
</tr>
<tr>
<td>A (n=14)</td>
<td>25</td>
<td>24</td>
</tr>
<tr>
<td>B (n=12)</td>
<td>20</td>
<td>34</td>
</tr>
<tr>
<td>C (n=10)</td>
<td>15</td>
<td>25</td>
</tr>
<tr>
<td>D (n=11)</td>
<td>19</td>
<td>27</td>
</tr>
<tr>
<td>E (n=12)</td>
<td>17</td>
<td>30</td>
</tr>
<tr>
<td>F (n=13)</td>
<td>19</td>
<td>23</td>
</tr>
<tr>
<td>G (n=6)</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>H (n=13)</td>
<td>20</td>
<td>31</td>
</tr>
<tr>
<td>I (n=14)</td>
<td>23</td>
<td>25</td>
</tr>
<tr>
<td>J (n=9)</td>
<td>15</td>
<td>21</td>
</tr>
<tr>
<td>K (n=12)</td>
<td>19</td>
<td>33</td>
</tr>
</tbody>
</table>

Formula for significant difference = \( \frac{\Sigma R_{11} - \Sigma R_{111}}{\sqrt{2n}} \sim N(0,1) \)
A. Tears placed in the antigen well are separated when an electrical potential is applied.

B. Antiserum is placed into a trough cut into the agar. Antiserum interacts with tear protein immunoglobulins forming precipitin lines.

C. Precipitin lines are stained and scanned with a densitometer.

* Adapted from Fudenberg, H.H.
IMMUNOELECTROPHORESIS

A. Immunelectrophoresis Slide

B. Diffusion of Antigen and Antiserum

C. Developed Slide
Figure 2

RADIAL GEL DIFFUSION

A. A predetermined volume of tears are placed in the antigen well.

B. Top view showing ring diameters produced by standard solutions of known concentration.

* Appended from Fudenberg, H. H.
RADIAL GEL DIFFUSION

A. Specific Antiserum Agar with Antigen Well

B. Completed Process

Ring Produced by Unknown

Rings Produced by Standards
Figure 3

1.)

The Abbott Biochromatic Analyzer 50 used for determining total protein concentration in tear samples.

2.)

Series of SMI Micro/Pettor micro pipettes used in measuring and dispensing fixed volumes of tears and Total Protein A-Gent substrate.
Figure 4
Top view of the multicuvette composed of 32 serially arranged cells of equal size.
Figure 5

Fixed volumes of tear fluid are drawn from the "microcap" micro-capillary tubes by placing the Micro/PettorR micropipette flush against the capillary tube and extracting 5 microliters.
Figure 6
Schematic of the operating principle of the Abbott Biochromatic R Analyzer 50.
Figure 7

Beckman R-100 Microzone Electrophoresis system used to separate total protein into component fractions.

Beckman RD-2 Duostat Regulated Power Supply.

Beckman R-101 Microzone electrophoresis cell.

Beckman R-110 Densitometer.
Figure 8

Schematic of Electrophoresis Procedure

1. Tears are collected from the eye using microcapillary tubes.

2. Tear proteins are separated into bands when placed in an electric field.

3. Membrane containing protein bands is run through a series of solutions to stain the separated protein fractions.

4. Transparent membrane containing protein bands is scanned with a densitometer to determine the relative amount of protein present in each band.
PROTEIN ANALYSIS OF THE TEARS

1. Sample
2. Electrophoresis
3. Dying and Fixing Baths
4. Photodensitometry

Amount of Protein
Figure 9

Densitometer scan showing the relative amount of protein present in the two bands.
Figure 10

Transparent membrane containing darkly stained bands of tear protein fractions. Identification of tear protein components is achieved by comparing their mobilities to those displayed by known protein standards.
<table>
<thead>
<tr>
<th></th>
<th>Lysozyme</th>
<th>γ Globulin</th>
<th>α Globulin</th>
<th>Albumin + α Globulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Albumin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>α Globulin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Tears</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>γ Globulin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Lysozyme</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 11 (a-k)

Graphs of hourly variation in total protein concentration for five days for subjects a-k.
TO TAL PROTEIN CONCENTRATION (G/dL)

SUBJECT "A"

HOUR OF THE DAY

8 AM 9 10 11 N 1 2 3 4 5 6 7 8 9 PM

- - - Monday
++- Tuesday
◊-◊ Wednesday
□-□ Thursday
X-X Friday
SUBJECT "B"

TOTAL PROTEIN CONCENTRATION (g/dl)

HOUR OF THE DAY

- Monday
- Tuesday
- Wednesday
- Thursday
- Friday
SUBJECT "C"

TOTAL PROTEIN CONCENTRATION (g/dL)

HOUR OF THE DAY

8 AM 9 10 11 N 1 2 3 4 5 6 7 PM

x—x Monday
++ Tuesday
◊◊◊ Wednesday
□□□ Thursday
x—x Friday
SUBJECT "D"

TOTAL PROTEIN CONCENTRATION (G/DL)

HOUR OF THE DAY

- Monday
- Tuesday
- Wednesday
- Thursday
- Friday
TOTAL PROTEIN CONCENTRATION (g/dL)

HOUR OF THE DAY

8 AM 9 10 11 12 1 2 3 4 5 6 7 8 PM

SUBJECT "G"

x-x Monday
++-+ Tuesday
--o Wednesday
--o Thursday
-x-x Friday
Figure 12 (a-k)

Frequency histograms of total protein concentrations for subjects a-k.
SUBJECT "A"

N = 75.00
\bar{X} = 0.56
\sigma = 0.11
SUBJECT "B"

N = 68.00
\( \bar{X} = 0.68 \)
\( \sigma = 0.12 \)

TOTAL PROTEIN CONCENTRATION (G/dL)
SUBJECT "C"

\[ N = 55.00 \]
\[ \overline{X} = 0.74 \]
\[ \sigma = 0.12 \]
SUBJECT "D"

N = 62.00
\( \bar{X} = 0.77 \)
\( \sigma = 0.13 \)
SUBJECT "E"

N = 70.00
\( \bar{x} = 0.78 \)
\( \sigma = 0.20 \)
SUBJECT "F"

N = 75.00
\( \bar{X} = 0.78 \)
\( \sigma = 0.20 \)

TOTAL PROTEIN CONCENTRATION (G/dL)
SUBJECT "H"

N = 70.00

\[ \bar{X} = 0.87 \]

\[ \sigma = 0.17 \]
SUBJECT "I"

N = 64.00
\( \bar{X} = 0.87 \)
\( \sigma = 0.21 \)
SUBJECT "J"

N = 69.00

\( \bar{X} = 0.92 \)

\( \sigma = 0.19 \)
SUBJECT "K"

N = 65.00

$\bar{X} = 1.11$

$\sigma = 0.46$
Figure 13

Frequency histogram for all total protein concentration measurements collected from this sample population. (N=732)
N = 732
\( \bar{X} = 0.81 \)
\( \sigma = 0.25 \)
\( \gamma_1 = 1.77 \)
\( \gamma_2 = 7.83 \)
Figure 14 (a-k)

Graphs of hourly mean total protein levels for subjects a-k. Range limits and meal times are overlaid.
SUBJECT "A"

TOTAL PROTEIN CONCENTRATION (g/dL)

MEAL 1

MEAL 2

MEAL 3

HOUR OF THE DAY

8 AM 9 10 11 PM 1 2 3 4 5 6 7 8 9 PM
SUBJECT "B"

TOTAL PROTEIN CONCENTRATION (G/DL)

HOUR OF THE DAY
SUBJECT "D"

TOTAL PROTEIN CONCENTRATION (G/DL)

HOUR OF THE DAY

8AM
9 10 11 12 1PM
4 5 6 7 8PM
SUBJECT "F"

TOTAL PROTEIN CONCENTRATION

HOUR OF THE DAY

8 AM 9 10 11 12 1 2 3 4 5 6 7 8 9 PM
SUBJECT "H"

TOTAL PROTEIN CONCENTRATION (G/L)

HOUR OF THE DAY

8 AM 9 10 11 PM 1 2 3 4 5 6 7 8 9 PM
Figure 15

Graphs of hourly variations in total protein concentration for one subject, (subject B), over the five-day sampling period.
TOTAL PROTEIN CONCENTRATION (G/DL)

HOUR OF THE DAY

DAY 1

N = 14.0
X̄ = 0.64
σ = 0.15
TOTAL PROTEIN CONCENTRATION (G/DL)

DAY 2

N = 14.0
\bar{X} = 0.65
\sigma = 0.06
TOTAL PROTEIN CONCENTRATION (g/dL)

HOUR OF THE DAY

\[ N = 13.0 \]
\[ \bar{X} = 0.72 \]
\[ \sigma = 0.15 \]
DAY 5

TOTAL PROTEIN CONCENTRATION (G/DL)

N = 13.0
\( \bar{X} = 0.74 \)
\( \sigma = 0.08 \)
Figure 16

Graph of hourly mean total protein concentrations for the entire population. Standard deviations are overlaid.
N = 732
\bar{X} = 0.81
\sigma = 0.25
Figure 17 (a-k)

Graphs of hourly variation in the proportion of each protein fraction.
Subject "A"

Fraction I
Fraction II
Fraction III

PROPORTION

HOUR OF THE DAY

8 AM 9 10 11 12 1 2 3 4 5 6 7 8 9 10 PM
SUBJECT "B"

PROPORTION
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00

HOUR OF THE DAY
8 AM 9 10 11 N 1 2 3 4 5 6 7 8 9 10 PM

Fraction I
Fraction II
Fraction III
SUBJECT "F"

PROPORTION

HOUR OF THE DAY

Fraction I
Fraction II
Fraction III
Subject "G"

Fraction I

Fraction II

Fraction III

8 AM 9 10 11 PM

HOUR OF THE DAY
SUBJECT "G"

HOUR OF THE DAY

PROPORTION

x -- x Fraction I
++ -- Fraction II
◊ -- ◊ Fraction III
SUBJECT "I"

- Fraction I
- Fraction II
- Fraction III

HOUR OF THE DAY

PROPORTION

0.00 0.10 0.20 0.30 0.40 0.50 0.60 0.70

8 AM 9 10 11 PM 12 1 2 3 4 5 6 7 8 9 10
PROPORTION

0.70

0.60

0.50

0.40

0.30

0.20

0.10

0.00

SUBJECT "K"

x-x Fraction I

+-----+ Fraction II

◊◊◊ Fraction III

HOUR OF THE DAY

AM 9 10 11 N 1 2 3 4 5 6 7 8 9 10 PM

0.70

0.60

0.50

0.40

0.30

0.20

0.10

0.00
Figure 18 (a-k)

Graphs of hourly concentration variation for total protein and each of the three separable fractions. Concentration is given in grams/dL.

d/L=100 ml.
CONCENTRATION (g/dL)

SUBJECT "A"

X---X  Total Protein
x-----x  Fraction I
+-----+  Fraction II
◇----◇  Fraction III

HOUR OF THE DAY
SUBJECT "C"

CONCENTRATION (g/dL)

HOUR OF THE DAY

8 AM  9  10  11  N  1  2  3  4  5  6  7  8  9  10 PM

Total Protein
Fraction I
Fraction II
Fraction III
SUBJECT "D"

CONCENTRATION (G/DL)

HOUR OF THE DAY

8 AM 9 10 11 N 1 2 3 4 5 6 7 8 9 10 PM

X—X Total Protein
x—x Fraction I
++ Fraction II
◊◊ Fraction III
CONCENTRATION (G/dL)

SUBJECT “E”

X—X Total Protein
x—x Fraction I
+—+ Fraction II
◊—◊ Fraction III

HOUR OF THE DAY
CONCENTRATION (G/dL)

X--X Total Protein
x--x Fraction I
++-+ Fraction II
◇◇-◇ Fraction III

SUBJECT "F"

CONCENTRATION (G/dL)

HOUR OF THE DAY

8 AM 9 10 11 M 1 2 3 4 5 6 7 8 9 10 PM
CONCENTRATION (G/DL)

X---X Total Protein
x---x Fraction I
++-+ Fraction II
◇◇ Fraction III

SUBJECT "G"

HOUR OF THE DAY

CONCENTRATION (G/DL)

0.10

0.30

0.50

0.70

0.90

1.10

1.30

1.50

8 AM 9 10 11 12 1 2 3 4 5 6 7 8 9 10 PM

HOUR OF THE DAY
SUBJECT "G"

CONCENTRATION (G/DL)

8 AM 9 10 11 12 1 PM 2 PM 3 PM 4 PM 5 PM 6 PM 7 PM 8 PM 9 PM 10 PM

HOUR OF THE DAY

X—X Total Protein
x—x Fraction I
++ Fraction II
◇◇ Fraction III
CONCENTRATION (G/dL)

XS X Total Protein
x—x Fraction I
+++ Fraction II
○○ Fraction III

SUBJECT "H"

HOUR OF THE DAY

CONCENTRATION (G/dL)

8 AM 9 10 11 12 1 2 3 4 5 6 7 8 9 10 PM

0.25 0.75 1.25 1.75 2.25 2.75
CONCENTRATION (G/DL)

SUBJECT "K"

X--X Total Protein
x--x Fraction I
++-- Fraction II
○--○ Fraction III

HOUR OF THE DAY

CONCENTRATION (G/DL)

HOUR OF THE DAY
Figure 19 (a-d)

Graphs showing changes in mean protein concentration during hydrophilic lens adaptation and again when adaptation was completed.
TOTAL PROTEIN CONCENTRATION (G/dL)

SUBJECT 1
Pre-Adaptation
5 days  N = 30
\( \bar{x} = 0.73 \)
\( \sigma = 0.11 \)

Adaptation
5 days  N = 30
\( \bar{x} = 0.68 \)
\( \sigma = 0.09 \)

Post-Adaptation
2 days  N = 12
\( \bar{x} = 0.71 \)
\( \sigma = 0.06 \)

HOUR OF THE DAY
TOTAL PROTEIN CONCENTRATION (G/dL)

SUBJECT 2
Pre-Adaptation
5 days  N = 30
      \bar{x} = 0.83
      \sigma = 0.18

Adaptation
5 days  N = 30
      \bar{x} = 0.79
      \sigma = 0.21

Post-Adaptation
2 days  N = 12
       \bar{x} = 0.85
       \sigma = 0.10

HOUR OF THE DAY

00 01 02 03 04 05 06 07 08 09 10 11 12 13 14 15 16 17 18 19 20 21 22 23
SUBJECT 3
Pre-Adaptation
5 days  N = 28
\( \bar{x} = 0.77 \)
\( \sigma = 0.13 \)
Adaptation
5 days  N = 27
\( \bar{x} = 0.75 \)
\( \sigma = 0.17 \)
Post-Adaptation
2 days  N = 12
\( \bar{x} = 0.76 \)
\( \sigma = 0.14 \)
SUBJECT 4
Pre-Adaptation
5 days N = 30
$\bar{x} = 1.12$
$\sigma = 0.49$

Adaptation
5 days N = 30
$\bar{x} = 1.00$
$\sigma = 0.22$

Post-Adaptation
2 days N = 12
$\bar{x} = 1.05$
$\sigma = 0.29$

TOTAL PROTEIN CONCENTRATION (G/dL)

HOUR OF THE DAY
Figure 20

Linearity of the Abbott Bichromatic Analyzer 50R

dL = deciliter = 100 ml.
INSTRUMENT READING (G/DL) vs. TOTAL PROTEIN STANDARD CONCENTRATION (G/DL)