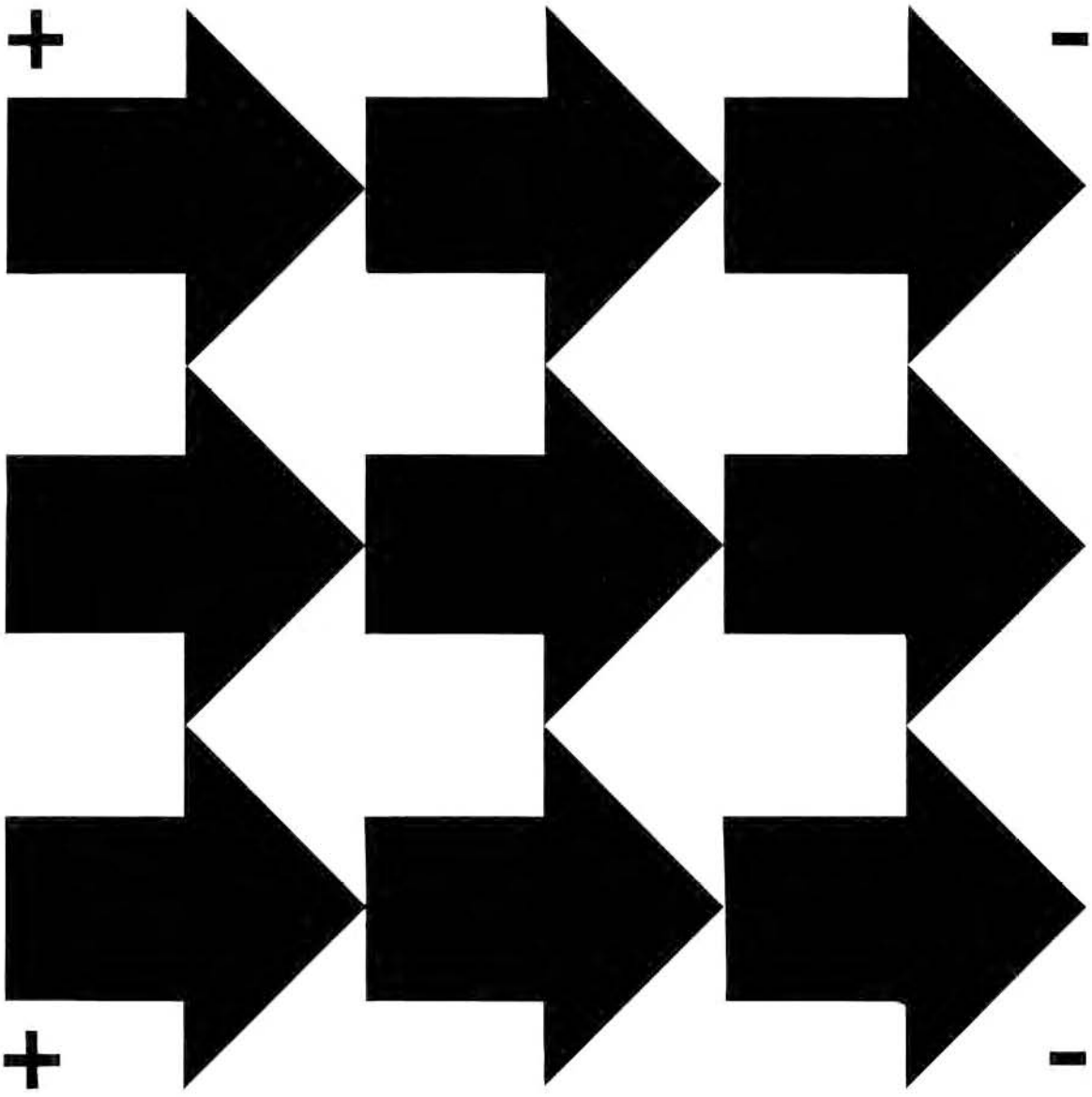
The Theory of Electrophoresis



HELENA
LABORATORIES

Electrophoresis

Electrophoresis is perhaps the most powerful tool a researcher or clinician has to monitor life processes. It is the primary method used to analyze proteins and since proteins are literally the very building blocks of life itself, their detection and analysis is essential to any study of living processes. The most dramatic use of electrophoresis is as a diagnostic tool. As such, it offers the clinician a vast array of knowledge that he is just beginning to understand. The use of protein electrophoresis with specific staining techniques such as those for lipids, glycogens, and enzymes have opened broad new horizons. The combination of immunoprecipitin reactions in conjunction with electrophoresis has given birth to a whole new area of science. Although progress in the last 15 years far outmodes the previous 150, the science is an embryo. Its future is a key to longer, healthier and more productive life.

Electrophoresis is simply the movement of charged molecules in an electric field. Three ingredients are necessary; (1) an electric field, (2) a charged particle, and (3) a medium in which the movement may occur. A Russian physicist, Reuss, first described the electrokinetic movement of colloids in 1809. L. Michailis named the migration of colloidions in an electric field "electrophoresis" and the terminology has been used since.

A. Tiselius is generally considered the modern day father of electrophoresis. His "moving boundary" electrophoresis of serum proteins in a U-tube greatly increased electrophoresis potential. Moving boundary electrophoresis is commonly called "free electrophoresis" since it occurs strictly in a liquid without the benefit of a solid matrix.

P. Konig, in 1939, reported using electrophoresis on paper strips saturated with an electrolyte to isolate a yellow pigment from snake vemon and thus the beginning of a form of electrophoresis which utilized a solid matrix to hold the electrolyte. Tiselius suggested the name "zone electrophoresis" to incompass all forms of electrophoresis on a solid support media and this form of electrophoresis has become highly used.

Any solid material which can absorb or hold an electrolyte is a potential media for zone electrophoresis. Paper was the first used, and is still popular today. Others, including agar gel, starch, acrylamide gel and cellulose acetate have proven successful since. The major advantage of zone electrophoresis lies in the stabilization of the migration, consequently the equipment and technique are much simpler.

Movement through cellulose acetate is similar to that through all other porous materials. It depends primarily on the nature of the charged particle, the character of the buffer, and the intensity of the electric field.

The first criteria for the sample to be electrophoresed is that it carry or be induced to carry a charge. Proteins are amphoteric, like amino acids, and may be charged positively or negatively depending on the pH of the ambient solution. If we consider a protein as a long chain of amino acids, the net charge on the protein is more easily described.

$$NH_3^+-R-COO^- \qquad (1)$$

Equation (1) represents a neutral (no charge) amino acid. The pH at which negative and positive charges are equal is the "isoelectric point (pI)". However if this amino acid is subjected to either more positive or negative ions (pH change of the ambient solution), the net charge on the group will become negative or positive.

$$NH_3^+-R-COOH \overset{H^+}{\leftarrow} NH_3^+-R-COO^- \overset{OH^-}{\rightarrow} NH_2-R-COO^- + H_2O \qquad (2)$$

An excess of positive ions (H^+) neutralizes the negative charge leaving a net positive charge on the group. Conversely, an excess of negative ions (OH^-) leaves a net negative charge on the group. Positively charged protein molecules exhibit more adsorption than do negatively charged proteins; therefore, negative charges are almost always used when electrophoresing proteins.

On cellulose acetate, the net charge on the protein is almost exclusively responsible for its proximity to other proteins. Molecular weight and the shape of the protein are negligible considerations. Alpha-2 macroglobulin with a molecular weight of almost 1,000,000 and a pI of 5.9 moves faster than haptoglobin with a molecular weight of 100,000 and pI of 6.1 in an alkaline buffer.

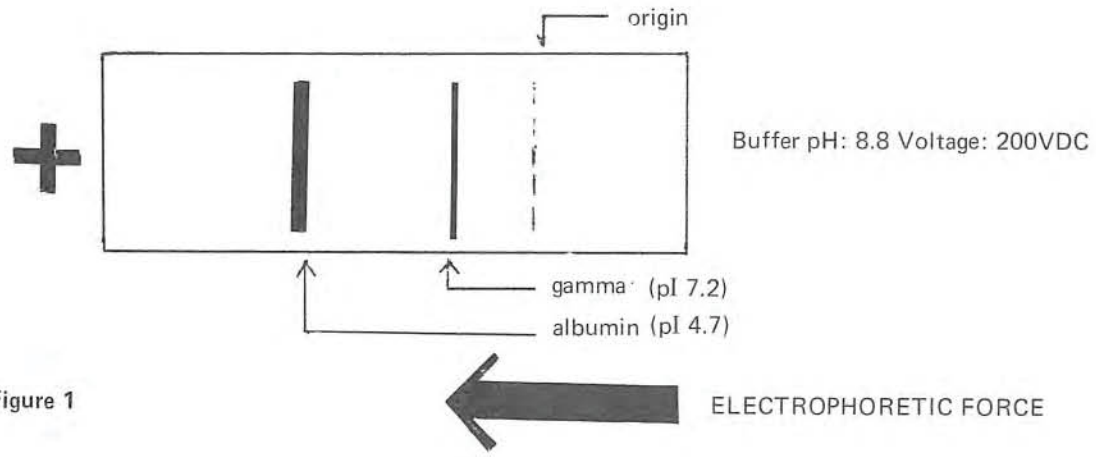


Figure 1

Buffers with a pH over 8.0 are generally used for protein electrophoresis. In an 8.8 pH buffer, albumin with a pI of 4.7 has the greatest net charge and gamma with a pI of 7.2 the least net charge. If these two proteins could be electrophoresed with mobility completely dependent on charge, albumin would have almost 2.6 times the mobility of gamma (see figure no. 1). Although net charge will determine the proximity of the protein, there are many other factors influencing the mobility.

Electro-osmosis, evaporation with resulting capillary movement, temperature, voltage, current, and ionic strength greatly contribute to the movement of proteins. Considering the nature of the matrix, gellation of the media, porosity, pore size and pore distribution, acetyllation, residual solvents and wetting agents in cellulose acetate, it is ludicrous to present a formula or group of formulas to explain and predict movement. Movement is at least as varied as the variety of cellulose acetates and that variety is infinite.

IONIC STRENGTH

Some influences can be predicted and should be given further consideration. The ionic strength (i.s.). of the buffer greatly influences the velocity of movement. As the ionic strength is lowered, the movement is greatly increased and the fractions become more diffused. As the ionic strength is increased; the movement is greatly reduced and the bands are held much tighter. In practice, ionic strengths from .01-0.1 have worked the best and a good starting point for any protein separation is 0.05. Amperage is also directly proportionate to ionic strength and this is an important consideration when choosing the buffer ionic strength.

$$\text{i.s.} = \tfrac{1}{2}\Sigma\, CZ^2 \qquad (3)$$

Σ = Summation of all ions
C = Molarity
Z = Ionic Charge

VOLTAGE – AMPERAGE – HEAT – TIME

Voltage is the driving force of electrophoresis, or the difference of potential. As the voltage is increased, the electrophoretic velocity of movement is increased proportionately. However, amperage is also increased proportionately and the resulting heat build-up is generally the limiting factor for volts or amps.

$$\text{Heat} = \text{Volts} \times \text{Current} \times \text{Time} \qquad (4)$$

Generally, sophisticated methods of dissipating heat are not used with cellulose acetate, therefore voltages do not exceed 500 volts or 60 volts/lineal cm. Current is generally limited to no more than 1 mA/sq. in.

Mobility is increased with higher temperature, but proteins are easily denatured and care must be taken not to exceed 50 degrees C. Higher temperatures lead to greater evaporation, which increases ionic strength in the strip since the buffer salts do not evaporate. Higher temperatures also increase buffer movement through the media as vacated pores are filled with buffer through capillary attraction from the buffer reservoirs.

The ionic strength change, which is more prominent along the edges of the cellulose acetate strip due to the increased evaporation surface area and buffer movement filling these vacated pores, generally results in unwanted artifacts. Temperature for these reasons is generally kept at room temperature or lower, even though faster movement is possible at higher temperatures.

Longer running times merely increase the opportunity for artifacts; since heat, buffer movement and free diffusion are compounded with time. It is generally preferable to keep running time to a minimum. These conditions for electrophoresis can only be empirically obtained. They are greatly interrelated and should not be considered separately.

BUFFER MOVEMENT – ELECTRO-OSMOSIS

The main advantage of zone electrophoresis over moving boundary electrophoresis is the relative stability of the buffer. However, there still remains a degree of movement which greatly affects the pattern.

Free diffusion (Brownian Movement) is still present although greatly reduced due to the solid matrix. The matrix itself, however, acts as a capillary and as evaporation occurs, the vacated pores are promptly filled from the buffer reservoirs.

The major buffer movement is due to electro-osmosis. Cellulose acetate contains polar groups, hydroxy (OH^-) and acetyl (CH_3COO^-) which become charged when subjected to a difference in potential (voltage). These negatively charged groups have a tendency to move towards the anode. However, they are stationarily bound to the cellulose groups. Since a force does exist in one direction, according to Newton, an equal force must be exhibited in the opposite direction. This "reaction force" is made up of buffer moving towards the cathode.

Again consider our protein solution of albumin (pI 4.7) and gamma (pI 7.2) in an alkaline buffer (pH 8.8). Both are negatively charged and should be attracted to the anode. However, the endo-osmotic force is generally greater than the electrophoretic force on gamma; and very frequently gamma will move towards the cathode.

ELECTRO-OSMOTIC FORCE

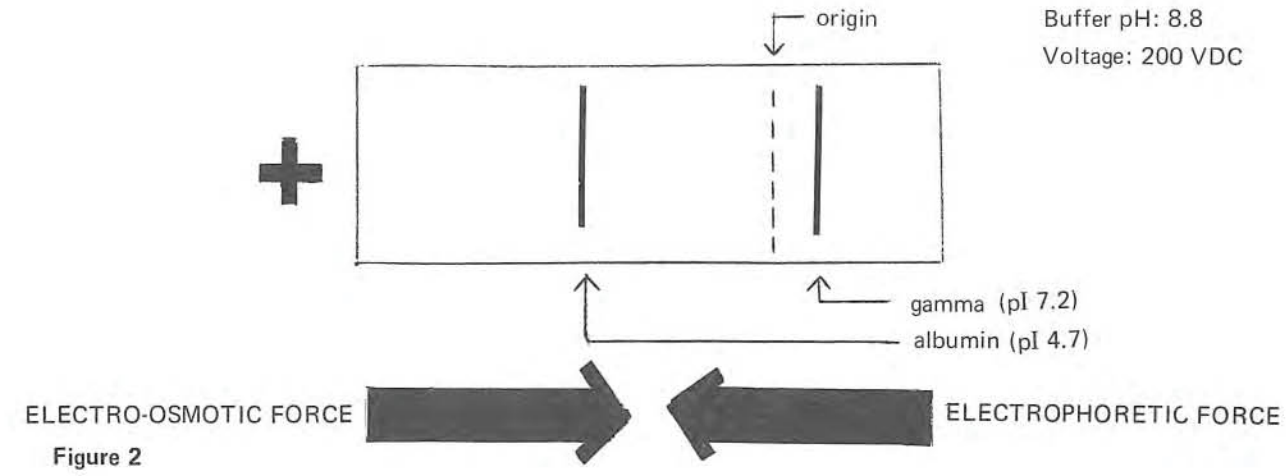


Figure 2

Indeed it is possible to reach a point on the strip where these two forces are equal and the resulting protein movement is nil. The protein literally reaches a point of equilibrium.

CELLULOSE ACETATE MATRIX

The different types of cellulose acetate electrophoresis media are infinite and this is why no two commercial products are identical or for that matter even closely similar. What might work well on one, may be completely inadequate on another.

There are two major factors responsible for the infinite variety of cellulose acetates possible. First, there is the length of the cellulose chain which could be anywhere from a few to millions of cellulose molecules and secondly the degree of acetyllations of the cellulose (0-44%).

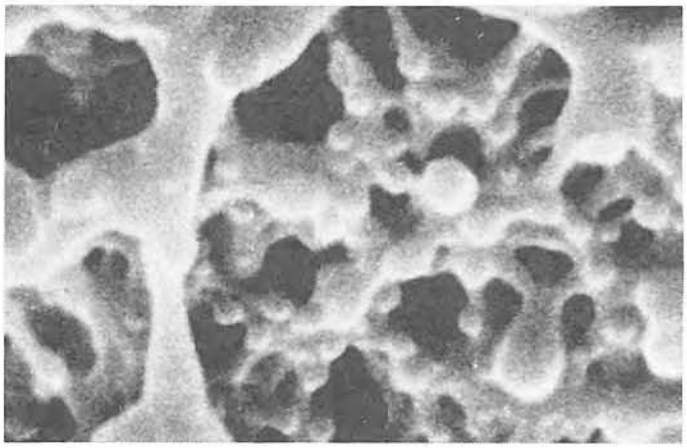
Other variables range from pore size (50 A - 10 u), pore distribution (variety of different pore sizes) and porosity (20-80%) or the volume of pores compared to the solid matrix. Gellation, chain coiling, type and degree of wetting agents and residual contaminents are even further considerations.

Cellulose acetate for electrophoresis is a uniformly formed matrix which would resemble the structure of a sponge or honeycomb if expanded several thousand times. The cellulose acetate, depending on its acetyl content and chain length, can be extremely inert and strong, or highly fragile and easily attacked by solvents. Gellation can be as great as 6% or as little as .1%.

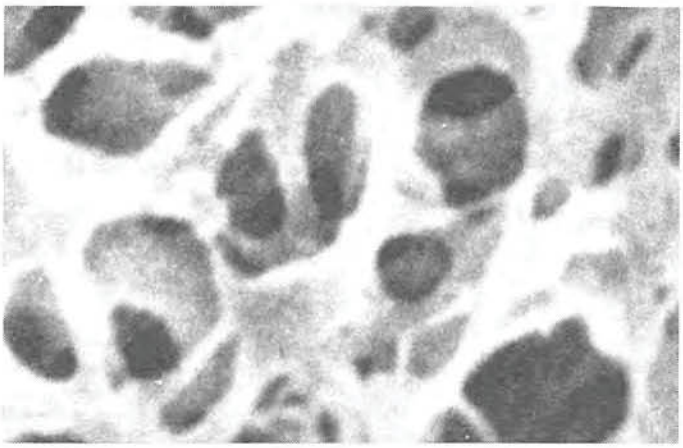
To expect a given set of conditions on two different cellulose acetates to produce the same results is like expecting two identical sets of fingerprints from different people. Unfortunately, characteristics which make cellulose acetate a better electrophoretic support media, generally make it more difficult to handle.

Cellulose acetate for electrophoresis is a spongy-like matrix of relatively uniform pores in random order. In use, these pores (80% by volume) are filled with buffer. Water molecules attached to the cellulostic structure both lubricate the pore openings and depress buffer movement.

PICTURE TITAN III 5,000



PICTURE CELLULOSE ACETATE 10,000 X



Titan III Cellulose Acetate Plate

There are two major distinguishing factors between Titan III Cellulose Acetate Plate and other cellulose acetate media. The primary one is the degree of gellation for Titan III. On Titan III, approximately 6% of the cellulose acetate molecule is water, while the degree of gellation on other cellulose acetates is only 1/3 as great.

This greater gellation results in a matrix much easier traversed by a charged molecule. One might consider the matrix better lubricated. It is the equivalent of sliding the palm of your hand over a dry surface vs. a wet surface. The result is much faster movement on Titan III when compared to other cellulose acetates.

The higher degree of gellation also affords better resolution. Resolution is decreased by diffusion and buffer movement in the matrix of the cellulose acetate. Imagine a protein molecule in this matrix. Its own Brownian Movement and continual buffer movement by electro-osmotic and capillary action cause constant diffusion. Of course these forces would be greatest in a free solution and they are greatly decreased when confined to the porous structure of any solid matrix. Cellulose acetate gellation or water molecules attached to the solid cellulose chains act as buffers to greatly reduce this diffusion. Titan III with 3 times the gellation of ordinary cellulose acetate, of course, has the greatest "baffling effect" and fractions are not subject to nearly as much diffusion. The end result is tighter bands or higher resolution.

From a practical point of view, cellulose acetate is not as easy to handle as paper. As previously discussed, the range of handling characteristics for cellulose acetate can be very broad. Most electrophoretic cellulose acetates are flimsy when wet although still easily handled. However, as the acetate is transformed into an optically cleared film, it becomes extremely difficult to handle and is subject to easy and varied distortion.

Titan III Cellulose Acetate Plate is composed of two layers. One is cellulose acetate and the other is a very inert polyester plastic sheet, Mylar. This Mylar supportive layer makes the cellulose acetate much easier to handle throughout the entire electrophoresis procedure, especially during the "clearing" step.

TITAN III CELLULOSE ACETATE PLATE (expanded side cut-a-way view)

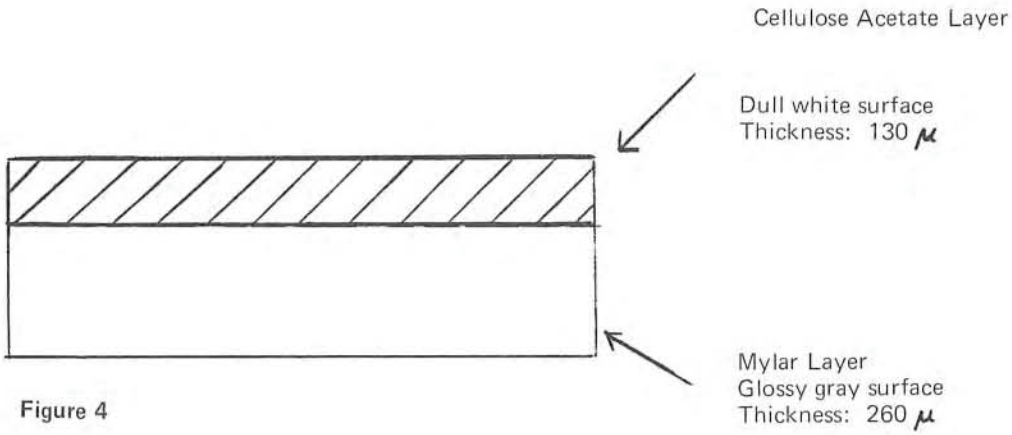


Figure 4

The Mylar layer also offers another advantage. It restricts evaporation during electrophoresis to only one surface, reducing the evaporation rate to about 1/2 that of ordinary cellulose acetate where evaporation occurs from two surfaces. This factor alone substantially reduces buffer movement through the cellulose matrix with, of course, a corresponding reduction of diffusion.

Titan III Cellulose Acetate Plate has the potential for better resolution than any possible on regular cellulose acetate. Conditions might have to be varied considerably to fully exploit the media. Up to 15 fractions for human serum proteins may be obtained with Titan III. Better detail for lipoproteins and hemoglobins has been well established. Any size plate is available and over 10 different sizes are routinely stocked.

Practical Aspects of Technique

It's nice to understand the theory of electrophoresis; however, much more important is making the procedure work. There are some cardinal "do's" and "dont's" and many "maybe's".

HANDLING THE STRIP:

Many papers suggest that cellulose acetate should not be touched by human hands and must be handled via forceps or some other manner at all times. This is completely ridiculous. Cellulose acetate, in fact, should be handled with hands wherever possible. It is by far the easiest and most practical way of manipulating the strips. Just be sure your hands are relatively clean and if you happen to spill a few drops of serum on your finger tips, clean them before handling the strip. Titan III plate can easily be supported from its edges, even after processing through the "clearing" procedure.

WETTING AND SOAKING THE STRIP:

All that is important is that after wetting, you have a uniform media. Regular cellulose acetate and Titan III are wetted in different manners. Regular acetate is floated on the buffer surface and allowed to wet. Titan III must be slowly dipped into the buffer. If the wetting process is carried out too quickly, the strip will at worst blister, making it completely useless; and at best trap air ridges which might distort protein movement through the layer. Slow wetting by dipping or even better by gradually raising the buffer level is recommended. To insure that the strip is in complete equilibrium allow the strip to soak for 20 minutes prior to use.

Soaking does alter the results slightly and a uniform soaking time should be established. All cellulose acetates contain wetting agents to aid the ordinary hydrophobic matrix in buffer absorption. These wetting agents have little effect on protein movement, with the exception of drastically changing lipoprotein mobility. Prolonged soaking times will dissipate the wetting agents and affect lipoprotein movement.

Prolonged soaking times also relax the coiled structure of the cellulose chain, causing several changes in the layer. First, the layer becomes larger, holding more buffer (ions), therefore generating more current and heat during the run. Secondly, the matrix itself has a different pore structure, offering a different resistance to protein movement. From a practical point of view, soak your strips for a relatively uniform period. A few hours makes little difference.

BLOTTING THE STRIP:

This, one would think, would be an unlikely area to cause problems but in reality is where much poor electrophoresis starts. One must blot the strip well enough, so that the serum sample readily enters the porous matrix of the cellulose acetate, yet it is important not to vacate so many pores during the blotting that buffer reabsorption in the chamber will result in excessive and unwanted buffer movement in the strip.

Titan III cellulose acetate has a high affinity for buffer trapped in its pores and must be blotted firmly enough to remove all excess surface buffer and a small amount of "pore trapped" buffer. If the blotting isn't firm enough, the serum sample will not be readily accepted and will have a tendency to "run" on the surface leaving a broad application instead of a narrow application. It is not easy to over-blot Titan III and the blotting should be between two unused blotter sheets.

After blotting, the sample should be quickly applied and the Titan III strip hurridly placed in the chamber where the evaporation will be recessed and the strip will rapidly reabsorb buffer from the buffer reservoirs.

Between blotting the strip and placing it in the chamber, evaporation leads to many artifacts. First, the ionic strength changes in the strip itself. Secondly, the greater the evaporation, the more pronounced the buffer flow caused by capillary action when the strip is placed in the chamber. If more than one minute elapses between blotting and placing the strip in the chamber, it is likely that complete areas of dehydration or "air pockets" will be formed. These appear as "white blotches" on the strip. When this happens, be sure to allow the strip ample time to rewet in the chamber prior to electrophoresis. If electrophoresis were to occur immediately in a strip with "air pockets", the difference in resistance in these areas is so great, that the results would be very irregular.

SAMPLE APPLICATION:

"Results can only be as good as sample application." This is probably the only area left in electrophoresis that one might consider an "art" rather than a "science", but it is a very important one. Truly, some individuals never develop the skill and their results are easily recognized.

The objective when applying sample is to keep the application as narrow and uniform as possible, yet load enough sample to be easily and properly qualitated and quantitated. The geometry is simple:

APPLICATION WIDTH AFFECTS RESOLUTION

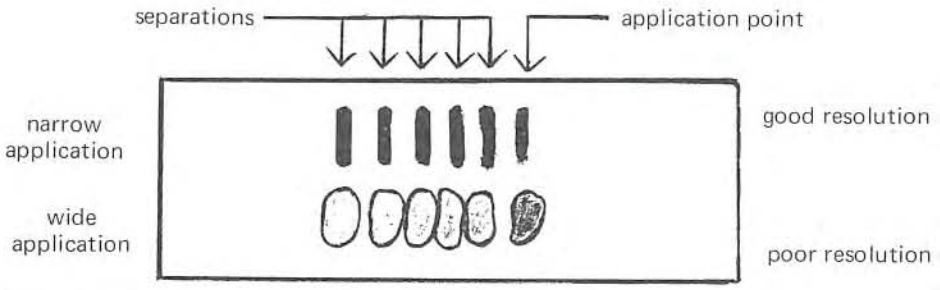


Figure 5

Narrow applications yield tight bands. Wide applications lead to excessive band overlapping, decreasing resolution.

Any applicator, technique, or method which leads to uniform, narrow applications is good, and any that doesn't should be changed. The first consideration must be given to the strip. It is absolutely essential that the strip be blotted firmly enough, so that sample will readily enter the strip.

Many types of applicators have been used on Titan III, but the Zip Zone applicator and companion Titan applicators have generally given the most consistently good results.

It's difficult to beat the Zip Zone Applicator for uniformity, since it subjects 8 samples to the identical amount of serum, drying time, etc. The delivery edges are designed so that sample is delivered from two sides greatly reducing the "dumbell" effect common in

many applicators. The technique is well presented elsewhere (See Zip Zone serum protein instructions), but it is worth emphasizing a few things. First of all, the applicator should be "primed" before use. This means that the first samples loaded should be wiped off with a hard, paper hand towel. "Priming" leaves a residual layer of protein on the applicator which makes the second "loading" much more uniform.

ZIP ZONE APPLICATOR DELIVERY TIP
(expanded side view)

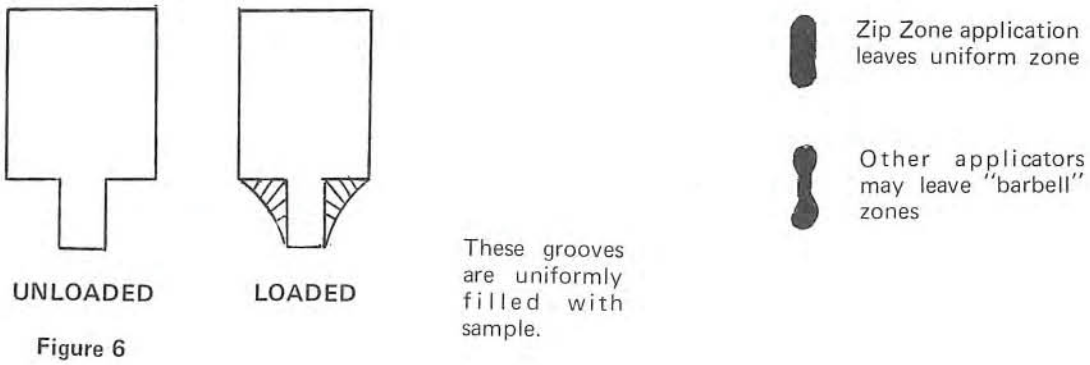
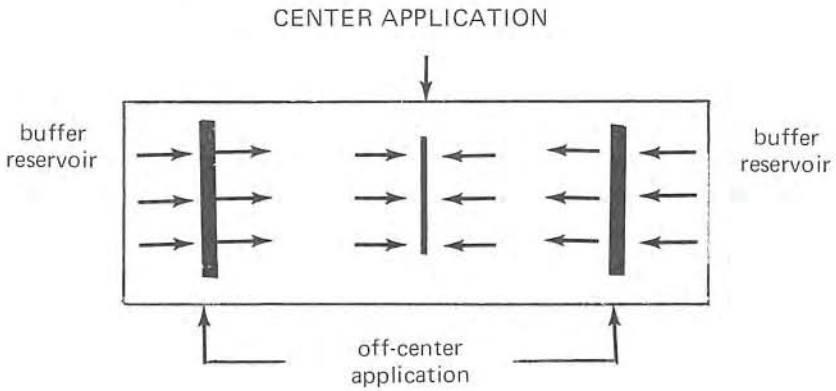


Figure 6

Once the applicator is loaded, apply the sample as quickly as possible. The minute amount of serum loaded on the applicator dries out rapidly and the resultant highly viscous film does not uniformly transfer to the cellulose acetate layer. It is good practice to have the strip blotted and in the aligning base prior to the final loading of the Zip Zone Applicator.

The point of origin has an effect on your results. Applications which are exactly or close to the center of the strip will in general offer better resolution. Proteins are subjected to buffer movement in the cellulose acetate matrix immediately upon application. Once the strips are in the chamber a great amount of buffer movement occurs from the buffer reservoirs towards the center of the strip as the strip rewets itself via capillary attraction.

CENTER APPLICATION OFFERS BETTER RESOLUTION



Buffer moving from the ends of the strip towards the center causes samples from off-center applications to move towards the center. This movement increases diffusion. Samples applied in the center have equal forces from both sides and retain their narrow origin.

By applying in the center, buffer movement from each side neutralizes the other's force. A center application may not always be feasible. If resulting artifacts interfere with either qualitation or quantitation, it may be necessary to move all proteins away from the point of origin. In this case apply only as close to the cathode as is necessary. Diffusion begins immediately with application, and therefore electrophoresis should begin as soon as possible after the application. For this same reason, the proteins should be fixed (stained) immediately after the electrophoresis run.

STAINING:

It is difficult, but not impossible, to make mistakes here. The only problem that occurs with any consistency is "understaining." The highly concentrated prteins, i.e. albumin, take much longer to stain than the lower concentrated proteins and it is possible to completely stain the globulins and not completely stain albumin. Since most methods of quantitation actually measure the "bound dye", understaining albumin will lead to low albumin values.

To ascertain the time required to complete the staining process, float the Titan III strip cellulose acetate side down on your stain. The background will rapidly assume the color of the stain, while the proteins won't yet have absorbed stain. The resulting pattern of white proteins on a stained background is called "Negative Staining." The staining time is complete once the proteins assume the same color as the background. Negative staining will also give you a quick preview of the separation.

Dye uptake difference among the different proteins is dependent not only on the protein, but also its concentration. The optimum area of dye uptake is discussed under "Quality Control" and "Quantitation."

CLEARING:

The polished cellulose acetate resin is clear, but the normal cellulostic structure of Titan III or any other cellulose acetate for electrophoresis is an amorphic one. This structure offers literally millions of surfaces for light reflection resulting in a brilliant white appearance for the cellulose acetate strip. Cellulose acetate strips are actually used to measure the degree of whiteness for other materials, since their appearance is pure reflected light.

The index of refraction for cellulose acetate is 1.47 and the strips may be rendered clear by subjecting them to any solution near this index of refraction. One of the early popular methods of clearing cellulose acetate was to wet it in oil.

Another method of clearing the strips is to break down the amorphic, porous structure of the strip into a film. This may be done by subjecting the strip to any solvent for cellulose acetate such as glacial acetic acid. The difficulty here is to clear the strip without introducing artifacts.

This type of clearing process is one where the strip must actually dissolve to work. Of course as it dissolves, it becomes a liquid rather than a solid and is difficult to keep in a uniform layer.

Most "clearing solutions" are a two solvent system. The "active" solvent, or the one which dissolves the cellulose acetate, generally evaporates slower than the "carrier" solvent, which initially only dilutes the active solvent to slow down its attack on the cellulostic structure.

While the strip is in the combination of solvents, it may still be handled. Once it is removed from this solvent bath and allowed to evaporate, clearing begins. As the "carrier" solvent evaporates, leaving a higher concentration of the "active" solvent, the cellulostic structure is gradually broken down into a film. The objective is to carry out the operation without the strip reaching too great a fluid state.

Titan III has a supportive layer, Mylar, which makes the strip easy to handle in the clearing process. It is generally beneficial to keep the cleared cellulose acetate bound to the Mylar for densitometry and storage. Cellulose acetate has a tendency to contract upon clearing and unless this is prevented, the cleared cellulose acetate will peel off the Mylar support. This isn't a great tragedy, since the cleared cellulose acetate for Titan III is just like any other cleared cellulose acetate, but it can be prevented.

In order to prevent this peeling, the cellulose acetate must not be allowed to contract during the clearing process. The clearing solution must be one with a high "active" solvent content and excess time should be allowed while the strip is in the clearing solution. This insures that the porous structure fully absorbs the clearing solution.

It is also beneficial to dissolve a small amount of cellulose acetate in the clearing solution. These extra cellulose acetate molecules help bridge gaps in the cellulostic structure of the strip as it clears, thus reducing contraction.

Quantitation

Once proteins have been fractionated by electrophoresis, there are several possible methods of quantitation. The most popular method is to stain the protein with a visible dye, then measure the intensity of the dye and correlate this intensity to the protein concentration. This method offers many advantages over alternative ones, but there are some pitfalls.

Other methods of quantitation are: (1) measurement of UV protein absorption, (2) measurement of fluorescently tagged protein, (3) measurement of radioactively tagged protein and (4) measurement of protein refractive indices.

Because the most common method, by far, is to visibly stain the protein, then measure the stain's intensity, it is the only one presented in detail. The other methods hold much promise for automating the complete electrophoresis procedure and you will undoubtedly be using one or more of them in future years.

PONCEAU S

Ponceau S has become the most popular stain for proteins. It is an anionic dye which reacts with the basic amino groups of the proteins in acid solution. It is convenient to use, and has become popular for many reasons: (1) It produces a vivid color at low concentrations of protein (detection level .05 ug). (2) It may be used simultaneously with a fixative agent like trichloroacetic acid, eliminating a separate fixing step necessary with other dyes. (3) It is readily washed from the background after the staining process, and (4) it is inexpensive, commonly available and easy to handle.

When using Ponceau S, or any other stain, the quantitative accuracy is dependent upon dye uptake, since the intensity of the dye is actually what is measured. Ponceau S reacts with the free amino groups of proteins. On a weight for weight basis, albumin has 1.42 more free amino groups than gamma globulin. One would then expect that albumin would absorb 1.42 times as much dye as gamma globulin on an equivalent weight basis. This hypothesis holds true when equivalent weights are used and conditions are such that all reactive sites are dye bound. However, for protein electrophoresis, albumin is in much higher concentration than gamma globulin and the above hypothesis doesn't hold true.

Different values will be seen on a given normal human serum sample with light, medium and heavy applications after staining with Ponceau S. The albumin value in this sample might be 70%, 60%, and 50% respectively.

With light applications, therefore light concentrations for all the fractions including albumin, nearly all the free amino sites will bind Ponceau S stoichiometrically. Without introducing a correction factor, albumin will be artifically increased while the globulins are decreased because albumin has more amino groups per gram weight.

With the medium application, the dye concentration of all the fractions is increased, but not proportionately. The concentration of the albumin band is such that many of the free amino groups are left unbound after the staining process while the amino groups for the globulins have completely reacted due to their lower concentration. The albumin fraction is so dense, that only the outer layers of the free amino groups bind dye. This outer layer of bound amino groups literally encapsulates the inner layers preventing them from dye binding. The net result is a pleasing one, however, since the values obtained for albumin and globulins are very near their true values on a gram weight basis.

The heaviest application resulted in the highest concentration of dye for each fraction, but the dye uptake became even more disproportionate. Albumin was only 50% of the total. The same thing has happened here as in the medium application. Only now the outer layers of bound amino groups have encapsulated even more free amino groups of albumin, while the globulins have not reached the point of not binding all their amino groups. The net result is one where the albumin value is lower than its true value.

The optimum, of course, is the medium application where true values are obtained by selectively depressing the dye uptake of albumin a proper amount. If the albumin concentration is controlled to the point where the optical density (OD) for this fraction is held between 0.7 and 1.2, values obtained for normal human serums will be near their true values. This is not an impossible task and is covered in more detail under "Quality Control" and "Practical Aspects of Technique."

DENSITOMETRY

Once the strip is stained, quantitation may proceed by two processes, densitometry or elution. Densitometry is such the preferred method that we hesitate to mention elution. The newer concepts of interpretation (See "Latest Concepts of Interpretation") require a graphic analysis. Peak heights, slope changes and slope proximity may all be used to expand the significance of electrophoresis. Densitometry is much easier and faster than elution. When technician time is considered, it is also much less expensive.

The theory and procedure for quantitation by densitometry is discussed in the "Quick Scan Manual." There are several areas that consistently cause errors in densitometry which are discussed under "Quality Control." It is preferable to scan cleared patterns rather than opaque ones since dye measurements follow "Beer's Law" much better. Occasionally, uncleared strips are scanned, but this is avoided when possible. Uncleared strips are scanned when an easy clearing method isn't possible. This is generally the case for lipoproteins and LDH Isoenzymes.

QUICK SCAN AND QUICK QUANT

Helena introduced the Quick Scan densitometer late in 1970 and in less than one year it had become the most popular scanner. It offers many advantages over conventional units (see "Quick Scan" and Evaluation of a New Scanning Densitometer") and in conjunction with the Quick Quant computer and Zip Zone apparatus, electrophoresis has reached the point where it may be considered for both preventive medicine purposes and as a broad-scale screening tool for diagnostic purposes.

ELUTION

Another method of quantitation is by elution. Although the elution is sufficiently accurate, it limits the degree of significance derived from the electrophoresis run and is more laborious. It does reduce the initial cost of doing electrophoresis and is recommended only when densitometry is not available for one reason or another.

There are two approaches to elution. One is to merely remove the dye and the other is to dissolve the cellulose acetate along with the dye, which is the best procedure for Titan II since the first method sometimes leads to turbid solutions.

The Proper Technique Is:

1. Carry out the electrophoresis run as is recommended for 1" x 3" Titan III. It's not possible to apply a heavy enough sample with Zip Zone.
2. Stain and destain the proteins as usual, but do not proceed to the clearing step. Leave the strips in the final 5% acetic acid rinse.
3. Label a set of test tubes for each fraction to be quantitated and leave them in a rack ready for the next step.
4. Blot the strip and cut out each fraction with a sharp pair of high-quality surgical scissors. Place the strip into the appropriately labeled tube (step no. 3).

ELUTION
serum proteins

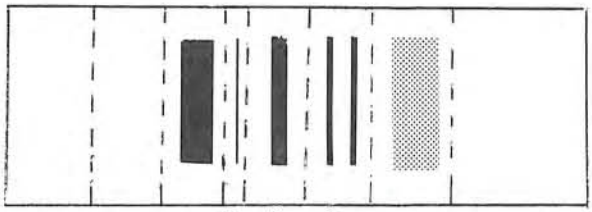


Figure 8

Cut each fraction, including a blank, as represented by the dotted lines.

5. Place the rack of tubes in an oven at 125 degrees C for ten minutes.
6. Remove the rack from the oven and add 2 ml of any good cellulose acetate solvent to each tube. Some better solvents are: demethylformamide (DMF), dimethylsulfoxide (DMSO), dioxane, glacial acetic acid and acetone.
7. Allow the cellulose acetate to completely dissolve. This may be speeded up by agitation or heating. If DMF, DMSO or glacial acetic acid is used, placing the rack back into the 125 degrees C oven for 5 minutes will greatly reduce the time required to dissolve the strips.
8. After the cellulose acetate has completely dissolved, measure the optical density (OD) of each fraction using the blank for a zero setting.

Example:

		OD
Albumin	-	.240
Alpha 1	-	.016
Alpha 2	-	.040
Beta	-	.040
Gamma	-	.064
Total		.400

To calculate the percentage:

$$\% = \frac{\text{Fraction OD}}{\text{total OD}} \times 100$$

Example:

$$\text{Alb} = \frac{.240}{.400} \times 100 = 60\%$$

To calculate gram %:

$$\text{Gram \%} = \frac{\text{Fraction OD}}{\text{total OD}} \times \text{total protein}$$

Example:

$$\text{Alb} = \frac{.240}{.400} \times 7.0 = 4.2 \text{ gm\%}$$

Quality Control

Quality Control for electrophoresis has been rather lax, compared to quality control in other areas of clinical chemistry. This is true because laboratories are unaware of various dye-binding characteristics, linear absorbance checks of densitometers, meaningful control serums and from a general unwillingness to put out the extra effort. Quality Control should be as routine for protein electrophoresis as it is for "sugars".

Good commercial serum controls are virtually nonexistent. All commercial controls must subject the proteins to procedures, like lyophilizing, chemical treatment and aging, which greatly reduce one's ability to resolve them. Commercial controls will invariably yield "fuzzier" and more diffuse bands than fresh serums. These bands have a much greater tendency to distort during the electrophoresis run.

Commercial controls are useful to the extent of reproducing their albumin values to within ± .2gm% and this will allow setting some other parameters. Reproducing their globulin values is another matter. The poor differentiation between fractions on the densitometric graph makes the peak selection become rather arbitrary. The values should be close to the controls, but don't be too disappointed if they are as much and (± .2gm%) different for gamma, beta and alpha$_2$.

I suggest first setting parameters to give accurate results (presented further in the chapter), then set aside a normal serum to serve as this year's control. First divide the serum into small alliquots. This can be quickly accomplished by filling standard microhematocrit tubes from a small vial serum. Fill them approximately 1/3 full and seal the ends in Critoseal, then freeze the entire year's supply. Once frozen, remove a tube and make at least 10 different runs on this sample. To establish realistic limits, use several strips and different technicians to make the runs.

After all the results are obtained decide the limits of variation for each fraction. We suggest, as maximum limits:

Albumin	± .20 g%
Alpha-1	± .05 g%
Alpha-2	± .10 g%
Beta	± .10 g%
Gamma	± .10 g%

Be sure that the conditions used to establish the values are conditions which lead to accurate results. You now have a freezer full of controls. Each working day, run an analysis on a fresh tube. Your results should fall within the limits you previously established and you can be assured that your procedure is at least reproducible.

DYE-BINDING:

The physical aspects of dye-binding more than anything else affect your results. This is presented in detail under "Practical Aspects of Technique". What you must do is establish conditions so that you obtain an albumin OD between .7 and 1.2 for the vast majority of your samples including the control. Your results will best correlate to true (accurate) values with this albumin density.

The density of albumin depends on many factors. Some are presented here to enable you to control this density to a great extent.

SAMPLE APPLICATION:

Albumin's end density is dependent upon application more than any other factor. The heavier your sample application, the denser the albumin fraction. Sample delivery systems from all manufacturers are at best mediocre. The Zip Zone Applicator and delivery system offer you the best chance of obtaining a reproducible sample application. A reasonably good technician with only several days experience should be able to reproduce sample delivery within ± 10%. It is indeed possible to reproduce sample delivery within ± 3% limits which is truly exceptional.

There are several other factors that influence the optical density of albumin and once a uniform sample delivery has been perfected, you might use them to maintain albumin's OD in the optimum range.

(1) Albumin is a narrower, denser band in high ionic strength buffers. High ionic strengths also have reduced migration characteristics which helps keep the band tight. Keeping all other conditions constant, the end albumin OD is lower in, say, .05 ionic strength (i.s) buffer than a .07 i.s. buffer.

(2) Higher voltages lead to denser bands. Lower volatages lead to less dense bands.

(3) Longer electrophoresis times lead to less dense fractions.

(4) The closer albumin moves to the anode, the less intense, its density. This is true because buffer movement due to electro-osmosis is strongest closer to the anode. Center applications can be used to take advantage of this effect, since albumin moves close enough to the anode to be influenced considerably, while the globulins do not and remain tight bands.

The sections "Electrophoresis Theory" and "Practical Aspects of Technique" and "Zip Zone Instructions" should be thoroughly read prior to a first attempt with Zip Zone and then reread at regular intervals afterwards.

UNIFORM FRACTION LENGTHS:

One of the common artifacts leading to inaccuracy is band length distortion. In this case, rather than all fractions having equal length or the same length as the application, some fractions may be "flared out" increasing their length while others are "condensed" reducing their length.

This, quantitatively, results in erroneously low values for the expanded fraction and high values for the condensed fraction.

These artifacts may be controlled by changing the buffer ionic strength and/or the voltage. Expanded bands are generally caused by too low an ionic strength or voltage. And condensed bands are generally caused by too high an ionic strength or voltage.

Sometimes a limited degree of "flaring" must be accepted. This is especially true in hemoglobin electrophoresis where the HbA_1 value is so high($> 95\%$) while the HbA_2 value is so low ($<4\%$).

UNIFORM FRACTIONS

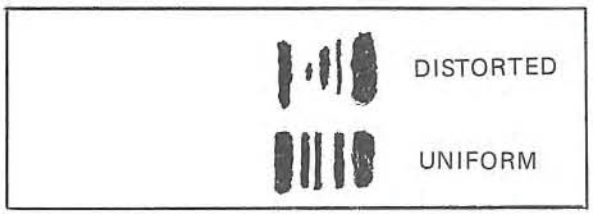


Figure 9 Albumin α_1 α_2 β γ

Voltage and buffer ionic strength must be geared to give uniform bands if accurate quantitative results are desired.

DENSITOMETER LINEARITY:

Another consideration in quality control is the linearity of the scanner. Your reproducibility is dependent upon your scanner's analog response. If your scanner's response isn't linear, it will reflect densities inaccurately. This has been a major source of error that has gone undetected in most laboratories.

The linearity of a scanner may be checked by scanning a series of known densities and plotting a graph. This was rather difficult until Helena introduced a single strip with increasing density sets that could be scanned just like a regular cellulose acetate strip. We have just made this optical "step wedge" available at $15.00 each and every lab should have one.

DENSITOMETRY – REPRODUCIBILITY:

Your densitometer should be periodically checked for reproducibility characteristics. Scan one pattern ten consecutive times trying to duplicate the scanning path as much as possible. You should allow little variation here. There are too many other potential error sources over which you have little control and this one can be controlled.

Poor reproducibility here is generally due to electrical drifting. This drifting may be in the analog, integration or lamp power circuitry.

One other aspect governingdensitometry reproducibility is your ability to select the identical scanning path on multiple scans. This is an error source not usually considered, but does exist and you should be aware of its limits. Make multiple runs on several different patterns. Be sure to completely remove the strip between each scan.

There are a few steps to help reduce the variation here. First, choose a scanning path free of any aberrations in the pattern. Be sure to align the light image (slit) so that it perfectly parallels the pattern's fractions. Avoid scanning areas of high and low density, "barbells," on the same pattern. If you have a pattern with "barbells," try to avoid scanning the "bell" portion if possible.

Slit height can affect results. Higher slits sample more of the band and theoretically lead to greater accuracy; however, most patterns are not uniform enough to consider high slits. If the slit is short enough, you can often scan a more uniform area of the pattern obtaining more accurate results.

A high slit may be the preferred slit when an automatic fraction detector is used like the Quick Quant. The larger slit reduces resolution somewhat, but also reduces pen noise lessening the chance of triggering extra peaks.

SERUM PROTEIN PATTERN WITH APPLICATION ARTIFACT SLIT SELECTION

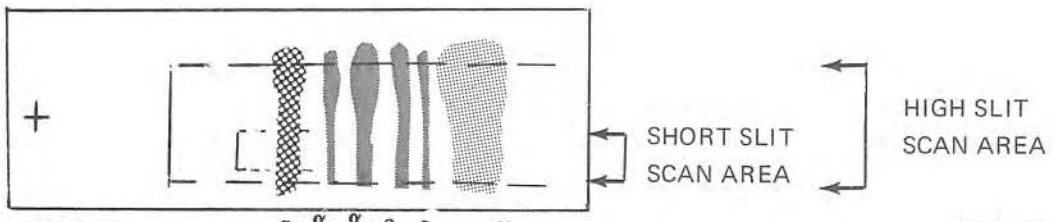


Figure 10

High slits scan a greater percentage of the pattern, but often must scan over artifacts in the pattern. Short slits are generally preferred since the best area of the pattern may be selectively scanned.

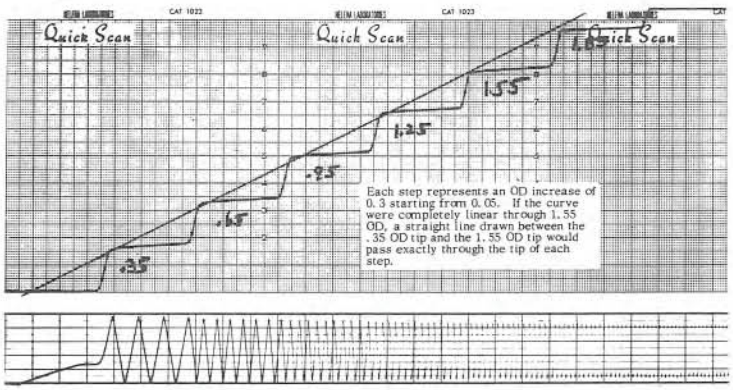


Figure 11

ESTABLISHING FRACTION OD:

As previously mentioned, it is necessary to obtain a final albumin optical density (OD) between .7 - 1.2 if accurate results are desired. Determining the OD of a given fraction is easy with an optical "step-wedge." Scan the "step-wedge" first at minimum "gain" if your scanner has a gain control. Now scan your pattern without making any "zero" adjustments. Position the maximum peak height of albumin or any other fraction in which you are interested, on the "step-wedge" graph so that it intersects a line drawn between the steps. The fraction OD is then established.

ALBUMIN DENSITY MEASUREMENT

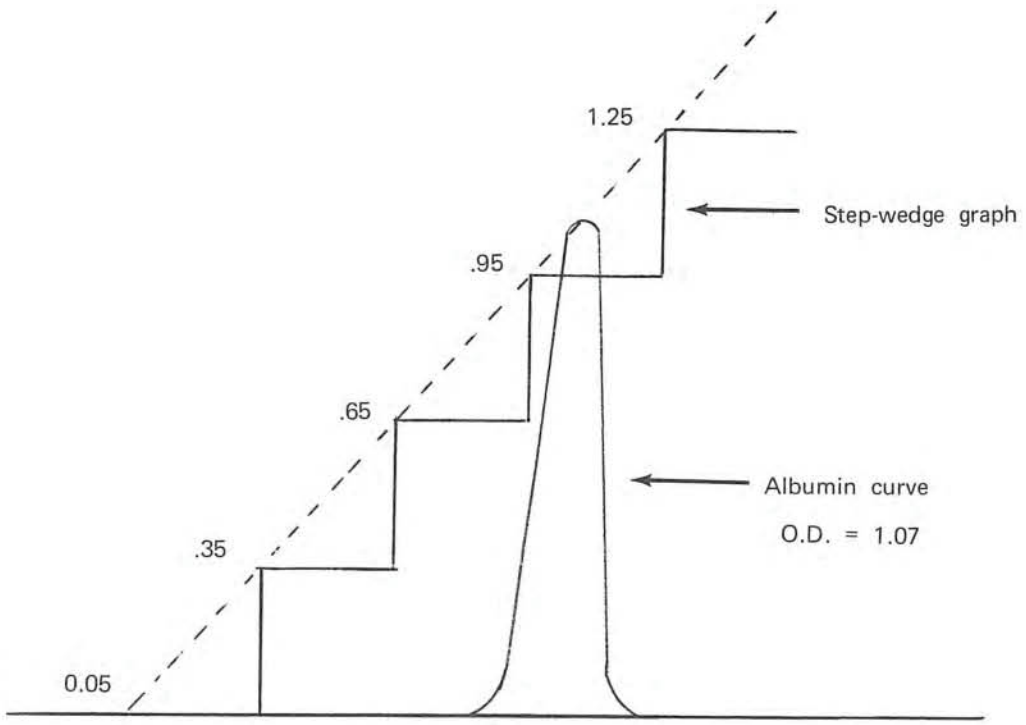


Figure 12

SAMPLE SERUM OR PLASMA:

Serums are generally preferred to plasma for electrophoretic analysis. The only difference between the two is that plasma samples exhibit one extra band, fibrinogen, which moves between beta-2 and gamma. A plasma electropherogram might eventually prove to be more useful than a serum electropherogram, since it would gain the added significance of a fibrinogen value.

Fibrinogen resolves well on Titan III Cellulose Acetate Plate and appears as a sharp band with little tailing to either side. Its normal mean is approximately 0.6 gm% and there is indication that part of gamma and beta are bound to fibrinogen in the plasma state. Whether or not the fibrinogen band interferes with IgA estimation or early monoclonal gammopathy detection has not been determined. More work is necessary to establish the relative merits of plasma electrophoresis.

HEMOLYSIS:

Hemolysis adversely affects electrophoretic analysis. A small amount will increase the alpha-2 value significantly since it binds with haptoglobin. Larger degrees of hemolysis will also increase the beta-1 value substantially since unbound HbA_1 moves in this area.

SAMPLE STORAGE – SERUM PROTEIN:

There is little change in protein fraction values when properly stored. The degree of resolution is decreased with time, but satisfactory results may be obtained under the following conditions:

Temperature	Good Results
Room (20-23°C)	up to 5 days
Refrigerator (2-5°C)	up to 30 days
Freezer (-20 to -40°C)	up to 5 years

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