Journal of Chromatography, 416 (1987) 265–280
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3562

COMPUTER ANALYSIS OF PROTEINS SEPARATED BY POLYACRYLAMIDE GRADIENT PORE GEL ELECTROPHORESIS

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(First received August 19th, 1986; revised manuscript received December 8th, 1986)

SUMMARY

A method for measuring lymph-to-plasma (L/P) protein concentration ratios obtained from protein fractions separated by polyacrylamide gradient gel electrophoresis is presented. A curve-fitting technique is used to decompose lymph and plasma electropherograms containing multiple components into individual components, eliminating protein–protein overlap regions. This allows the concentration of each component in the mixture to be measured accurately, yielding more precise estimates of L/P ratios. This technique consists of three phases. (1) Individual electropherograms are constructed for proteins of various sizes by taking a weighted average of measured electropherograms obtained from the two protein standards closest in size to the protein of interest. (2) Using these generated standard curves, the multicomponent lymph and plasma curves are decomposed into the least number of equally spaced components that yield a good fit. A linear least-squares method is used to do this. Each protein fraction is multiplied by the total measured protein concentration to provide a concentration for each component. (3) Finally, L/P concentration ratios of protein fractions with visible peaks were computed by applying an averaging technique to the equally spaced protein fractions. Plots of sheep lung L/P ratio versus protein size obtained in this manner were compared to L/P ratios obtained using a method of analysis which does not correct for protein overlap. The corrected L/P ratios showed less scatter than the uncorrected curves. Lung lymph data analyzed with the correction method indicated an increased lung microvascular permeability for large proteins following endotoxin infusion, whereas the uncorrected curves were too noisy to support this concept.

INTRODUCTION

Lymph-to-plasma (L/P) protein concentration ratios for proteins of different sizes are the primary experimental data on which porosity of the lung microvascular barrier is determined [1–3]. The accuracy of estimated transport parameters increases as the number of measured proteins increases. It is desirable then to separate as many proteins of different sizes as possible in plasma and lymph.

We currently use polyacrylamide gradient gel electrophoresis to separate lung lymph and plasma protein samples obtained from unanesthetised sheep under

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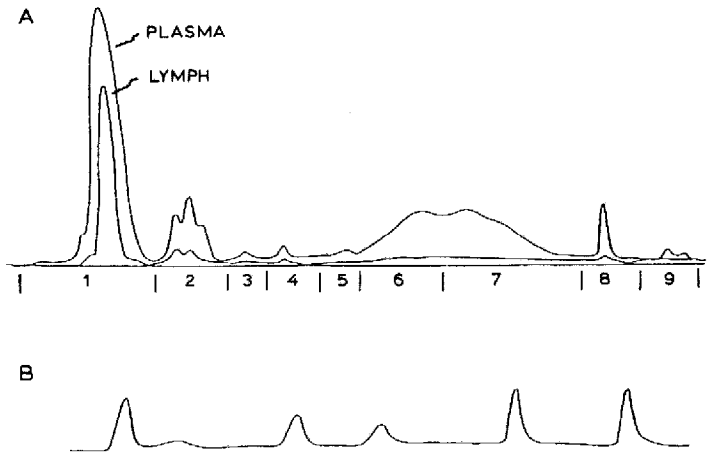

Fig. 1. Electropherograms of (A) sheep plasma and lung lymph proteins separated by polyacrylamide gradient gel electrophoreis and (B) the five protein standards used in our laboratory. Peaks (from left to right): albumin, LDH, catalase, ferritin, and thyroglobulin.

various experimental conditions. The electropherogram obtained for each sample is divided into nine defined fractions as shown in Fig. 1A and the area under each fraction is calculated after subtracting background variations resulting from density gradients in protein-free portions of the gel. Division by the total protein curve area gives the weight fraction of each component. Concentration of each protein fraction is obtained by multiplying the normalized area by the total protein concentration of the sample measured with a modified Biuret technique [4]. The same is done for the corresponding lymph or plasma sample and the L/P concentration ratio for that particular protein is computed.

A new method of analysis is proposed here, which will improve the accuracy of the calculated concentration for each protein fraction by correcting for the overlap of adjacent proteins in the electropherogram. Because the method is automated the speed of analysis can be greatly increased with greater accuracy than previously possible. Finally, the method will increase the number of protein fractions analyzed, thus providing more accurate estimates of capillary transport coefficients.

EXPERIMENTAL

Lymph and plasma samples were obtained from unanesthetised sheep under different physiological conditions (baseline, increased microvascular pressure and following intravenous (i.v.) infusion of *Escherichia coli* endotoxin). Proteins present in the samples were separated using gradient pore gel electrophoresis. Samples were placed on polyacrylamide (4–30%) gels (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.) using standard techniques [5]. A solution of Tris barbital–sodium barbital high-resolution buffer pH 8.8 was prepared and precooled to 5 °C. Gels in the pH range 8–9 are optimal for the separation of proteins which have isoelectric points within a pH range of 4–7.5 [6]. After 15 min of preelectrophoresis at 125 V, protein samples were loaded and a run-in period of 20

min at 75 V was performed. Electrophoresis was continued for 16 h at 125 V (constant voltage). Gels were stained with 0.5% Ponceau S in 7.5% trichloroacetic acid, destained electrophoretically in 7% acetic acid, and scanned between 450 and 510 nm with a TLC 525 scanning unit (Helena Labs., Beaumont, TX, U.S.A.). A mixture of five protein standards of known Einstein–Stokes radii (360–850 nm) were placed on each gel for comparison (Fig. 1B). The scanned samples were digitized using an analog-to-digital converter at the sample rate of 64 samples per s and a total sampling time of 23 s. The digitized data were collected on a PDP 11/34 computer and then transferred to a DEC-1099 computer for analysis. All programs for data analysis were written in FORTRAN-77, and IMSL Library subroutine OFIMA3 (IMSL, Houston, TX, U.S.A.) was used for linear least-squares parameter fitting.

ANALYSIS

The primary objective of this study is to obtain accurate L/P ratios for as many proteins of different sizes as possible. It requires accurate measurements of individual proteins in both plasma and lymph. It can only be accomplished by employing a least-squares curve-fitting technique which corrects each electropherogram for overlap by neighboring proteins. Such a technique requires two fundamental pieces of data: (1) the experimental electropherogram composed of a combination of all of the individual components and (2) electropherograms corresponding to each of the individual protein components.

The result of applying linear least-squares methods will be a computation of the relative amount of each component present in the mixture. First, each multicomponent electropherogram must be decomposed into its individual components. Since the number of desired components is always greater than the five measured standard components, each single protein standard needed for curve fitting must be generated from the five measured protein fractions. Second, a combination of these single components must be curve-fitted to the multicomponent lymph and plasma electropherograms to obtain the relative protein concentration for each of these single components. Finally, the L/P protein concentration ratios are calculated from the protein concentration fractions obtained from the curve-fit analysis.

APPLICATION OF THE LEAST-SQUARES TECHNIQUE TO PROTEIN SEPARATION ON POLYACRYLAMIDE GRADIENT PORE GELS

The least-squares technique is used widely in spectral analysis in which the parameter values of a theoretical model are selected so as to minimize the sum of the squared deviations between experimental data and calculated model values. Spectral data are most frequently recorded in a two-dimensional array (X,Y) in which the X values are the time intervals at which the spectral Y values are recorded, e.g. absorbance, counts, etc. The least-squares fitting technique will be used to decompose the multicomponent protein electropherogram into individual

protein components. In matrix form our problem is reduced to obtaining the least-squares solution to the matrix equation:

$$\mathbf{S}(n,m)\times\mathbf{C}(m)=\mathbf{T}(n) \tag{1}$$

where **S** is the array of $n\times m$ values containing each of the single standard digitized curves (input), n is the number of data points, m is the number of single protein fractions from which the multicomponent curve is to be constructed, **T** is an array of the n amplitude values containing the digitized experimental curve (input), and **C** is the array of m values containing the least-squares fitted parameters that minimize the sum of the squared errors, SSQ, between **T** and $\mathbf{S}\times\mathbf{C}$. These represent the distribution of equidistant single-component electropherograms which best reproduce the multicomponent curve.

By normalizing the individual and multicomponent curves by their respective areas, the additional constraint that the sum of all values of **C** must equal unity is imposed. Thus the value of $\mathbf{C}(m)$ can be written in terms of all other values of **C**. Eqn. 1 can be rewritten:

$$\mathbf{S}'(n,m-1)\times\mathbf{C}'(m-1)=\mathbf{T}'(n) \tag{2}$$

$\mathbf{S}'$ is the array of $n\times m-1$ values containing each standard curve normalized by its corresponding area from which the last (i.e., mth) standard curve has been subtracted, $\mathbf{T}'$ is the array of n amplitude values containing the experimental multicomponent curve normalized by its area from which the last standard curve has been subtracted, and $\mathbf{C}'$ is the array of $m-1$ values containing the concentration fractions of each of the $m-1$ protein fractions (output).

Therefore, by using available linear-least squares computer subroutines with $\mathbf{T}'$ and $\mathbf{S}'$ as input rather than **T** and **S**, the output of the program gives the concentration fractions of all but one of the protein fractions in which the multicomponent protein curves are decomposed. The concentration fraction of the final protein can be then found by subtracting the sum of all the others from unity.

To make use of the least-squares curve fitting method, the experimental digitized multicomponent curves, as well as the digitized single protein standard curves are needed as input. If the complex electropherogram is resolved into m protein fractions, m standard curves are needed for this analysis.

Many computer programs have been developed in the last few years which use the least-squares method for the analysis of spectra such as chromatography [7], infrared spectra [8], nuclear magnetic resonance [9] and electronic spectra [10]. However, little has been published on the use of the least-squares curve fitting technique in the separation of proteins by polyacrylamide gradient gel electrophoresis. Cole et al. [11] developed a computer program to analyze the profiles of chromatographic and isoelectric focusing curves which is also applicable to the polyacrylamide gel electrophoresis. However, it cannot be applied to pore gradient gel electrophoresis since gradient pore gel curves are not symmetrical and their shapes vary as a function of the gel concentration and protein molecular shape when the rest of the electrophoretic variables are kept constant.

When the number of components of a complex mixture and their location on

TABLE I

DATA OF AVAILABLE STANDARD GLOBULAR PROTEINS

Protein	Molecular weight	Einstein–Stokes radius (Å)	Amount (μg)
Albumin	67 000	36.0	40
Lactate dehydrogenase	140 000	41.6	48
Catalase	232 000	46.5	36
Ferritin	440 000	59.0	50
Thyroglobulin	669 000	85.0	76

the spectral curve are known, the least-squares problem consists of resolving the complex spectra into single components using a reference spectra library containing large numbers of either analytical or experimental digitized standard curves. For the present study the number of single components (proteins) present in the lymph or plasma protein sample is not known. However, the actual number of different proteins present is of little concern. Identification of proteins with different molecular sizes is the objective. Let m equally spaced protein fractions be defined in the protein mixtures such that each fraction represents a protein or set of proteins of nearly the same Einstein–Stokes radius. The electropherogram of the protein mixture can then be assumed to be a linear combination of the electropherograms attributable to each individual protein fraction.

GENERATION OF STANDARD CURVES

The five available pure protein standards (Pharmacia Fine Chemicals) used for the separation of lymph and plasma proteins by polyacrylamide gradient gel electrophoresis are given in Table I with their respective molecular weights, Einstein–Stokes radii and amount used in each sample. These proteins are used to find the molecular sizes of the protein fractions present in the multicomponent protein electropherogram. Based on Lasky's findings [12], the Einstein–Stokes radii are obtained from the empirical relationship between the electrophoretic mobility and the Einstein–Stokes radius shown in Fig. 2A and B and expressed by the relationship:

$$\log R = 1.548 - 0.60266 \log R_F \tag{3}$$

where R is the protein Einstein–Stokes radii in Angstrom units, R_F is defined as migration distance divided by migration distance of albumin, (i.e., the electrophoretic mobility relative to albumin).

β-Lipoprotein was electrophoresed once in our laboratory to obtain its migration distance on the gel. Fig. 2B shows that Lasky's relationship apparently does not hold for large molecules on these particular gels. Since only five standard proteins were used, only the shapes of these protein electrophoresis curves are known, leaving unresolved the problem of estimating the shapes of all other intermediate protein curves.

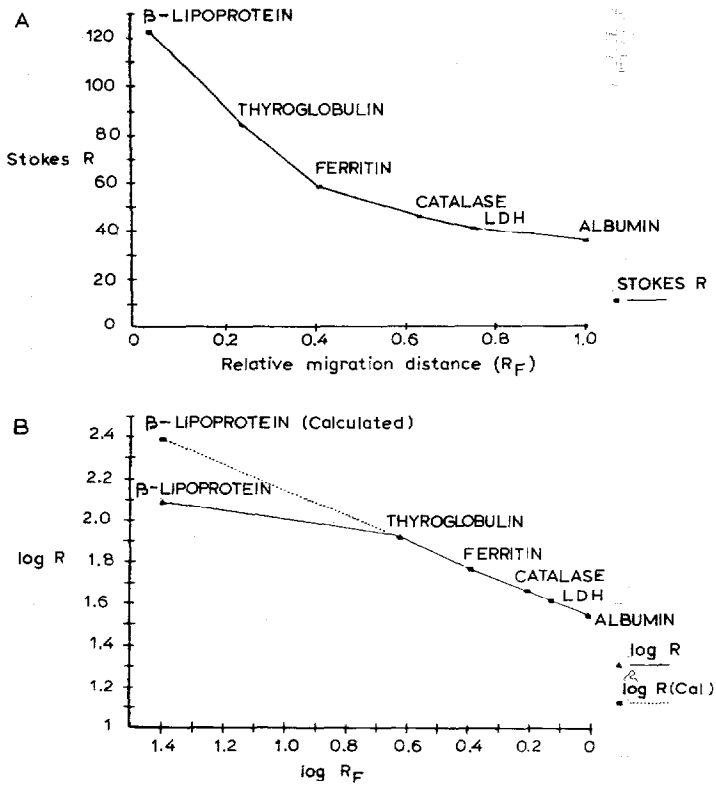

Fig. 2. (A) Einstein-Stokes radius versus electrophoretic mobility for protein standards. (B) Linear regression between the log of Einstein-Stokes radius and the log of the migration distance for the standard proteins (dashed line) and measured relationship (solid line).

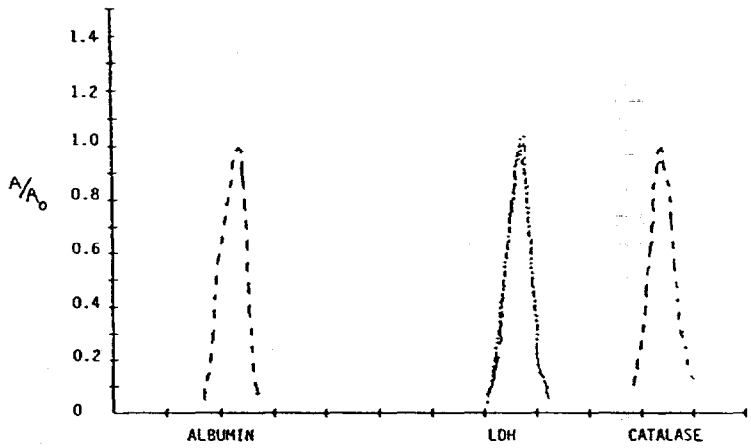

Fig. 3. Experimental LDH curve (dashed line) and 'ideal' LDH curve (solid line) generated by linearly combining the albumin and catalase experimental curves (dashed lines).

By looking at the electropherogram in Fig. 1B, which shows the five standard analog curves, a consistent trend can be observed. The shape of the curves changes from being asymmetrical in the direction of migration for small proteins (albumin) to being asymmetrical in the opposite direction for large proteins (thyroglobulin). The shape of a protein curve in a polyacrylamide gradient gel electropherogram is a function of the molecular size, shape, charge, electrophoretic time, voltage gradient, pH of the buffer, and the gradient of the gel. Under experimental conditions, the electrophoretic time and the voltage gradient as well as the gel gradient and the pH of the buffer are kept constant, and the charge effect is neutralized by having the protein migrating to its pore limit. Therefore, it can be assumed that the shape of a pure globular protein curve is only a function of its molecular size. Since proteins of different sizes migrate to different locations on gradient gels, it can be inferred that the shapes of a gradient gel electrophoresis curve for a pure globular protein can be determined from its migration distance along the gel.

If the above is true, then the migration of a pure protein with Stokes–Einstein radius between two of the protein standards should have a curve shape that is the linear weighted combination of the two experimental standard curves. Ideal standard curves for proteins smaller than albumin or greater than thyroglobulin are assumed to have the same shape as albumin (for smaller proteins) or thyroglobulin (for bigger proteins) since no linear combination of two known standard curves can be obtained for proteins with molecular sizes outside the available range. When albumin and catalase experimental electrophoresis curves were linearly combined to generate an 'ideal' lactate dehydrogenase (LDH) curve, it was found that the generated curve fitted the experimental LDH curve quite well ($SSQ/N=0.0065$). Fig. 3 shows the albumin, LDH and catalase experimental curves, and the generated 'ideal' LDH curves.

DECOMPOSITION OF MULTICOMPONENT ELECTROPHEROGRAMS USING A LINEAR LEAST-SQUARES TECHNIQUE

Having established the validity of estimating intermediate electrophoresis curves from proteins of known Einstein–Stokes radii, it is assumed that a limited number of standard curves can be used to decompose complex mixtures into individual components. The experimental data used in the least-squares analysis were obtained from gradient gel electrophoresis of lung lymph and plasma samples from two sheep placed on four different gels. Known protein standards were placed on each gel. Samples were collected during baseline conditions, after pulmonary pressure was increased by 15 Torr, and after injection of *E. coli* endotoxin.

Following the theoretical approach presented above, the five experimental standard curves and the lymph and plasma curves to be decomposed were normalized by their corresponding areas as shown in Fig. 4. To calculate the Einstein–Stokes radius of a protein or protein fraction from its migration distance on the gel, the empirical relationship between the relative migration distance R_F and the Einstein–Stokes radius given in eqn. 3 was used. The relative migration

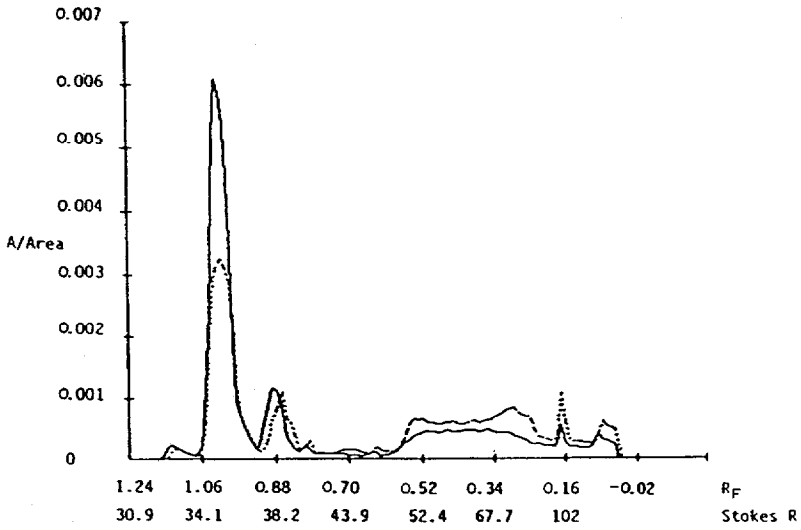

Fig. 4. Normalized (by area) plasma (dashed line) and lymph (solid line) protein electropherogram for sheep 1 during increased microvascular pressure (32 cmH_2O).

distance was calculated by dividing the migration distance by the migration distance of albumin.

In order to fit a combination of single protein standards to the multicomponent lymph and plasma electropherograms, we assume that the complex electropherogram is a continuous curve which at discrete locations contains proteins of unique size but varying concentration. Using this approach we systematically increased the number of proteins used in the analysis. Fig. 5A–C shows the experimental and fitted electropherograms for 18 (number of observed peaks), 50 and 100 assumed protein fractions. Equally spaced protein intervals were used in generating the last two curves. It can be seen that reasonable fits cannot be obtained when 18 or 50 components are assumed. However, when the mixture is decomposed into 100 individual components, the fitted curve is excellent.

Based on these findings, all subsequent curves were decomposed into 100 protein fractions. The normalized fractions are constrained so that the sum of all areas is equal to unity. Because of this restriction the regression routine occasionally adjusted some of the output values to be negative. A negative concentration fraction is meaningless and cannot be accepted. After looking into this more carefully it was found that the location of these negative concentrations corresponded to minima in the curves and the magnitudes were always quite small. Based on this observation, the negative protein concentration fractions were redefined to be zero.

CALCULATION OF THE L/P PROTEIN CONCENTRATION RATIOS

Using 100 concentration fractions, the L/P ratios were calculated for each component by dividing the lymph concentration fraction by the corresponding plasma

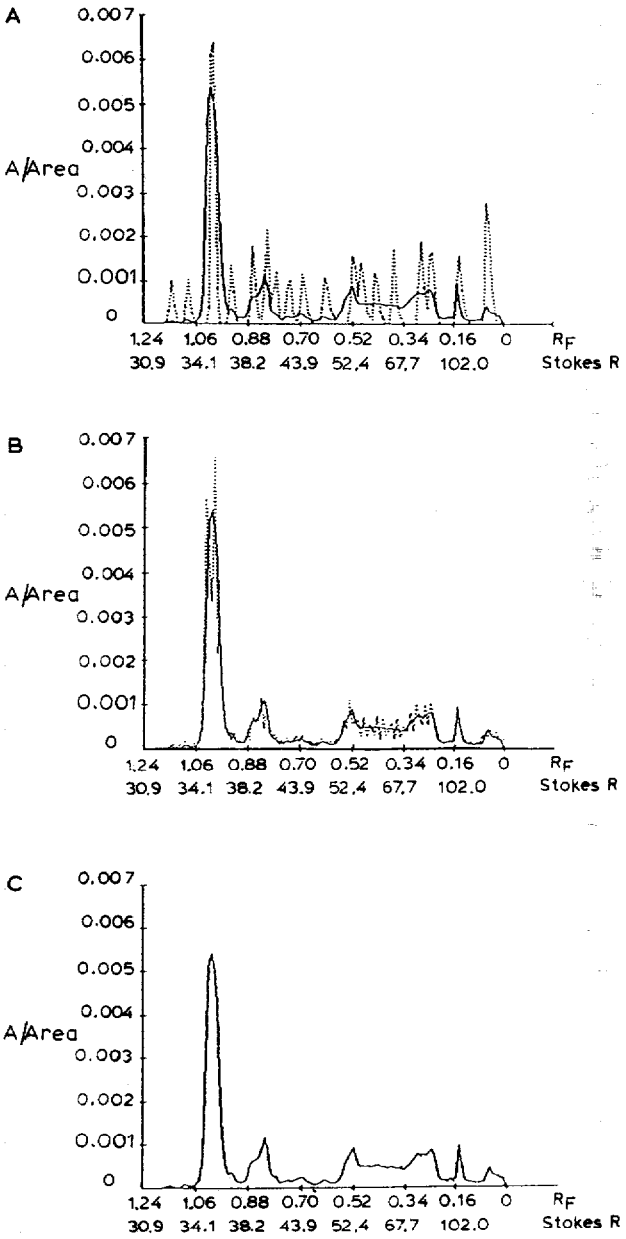

Fig. 5. Theoretical curves (dashed lines) obtained from the linear least-squares fitting of 18 (A), 50 (B) or 100 (C) discrete proteins to the complex plasma sample (solid line) for sheep 2 after injection of *E. coli* endotoxin.

concentration fraction and multiplying the ratio by the total L/P concentration ratio. The values so obtained were scattered, with some of the values greater than unity. Fig. 6A shows the 100 L/P ratios for samples from sheep 1 after pulmonary vascular pressure was elevated. From theoretical considerations [2] one would

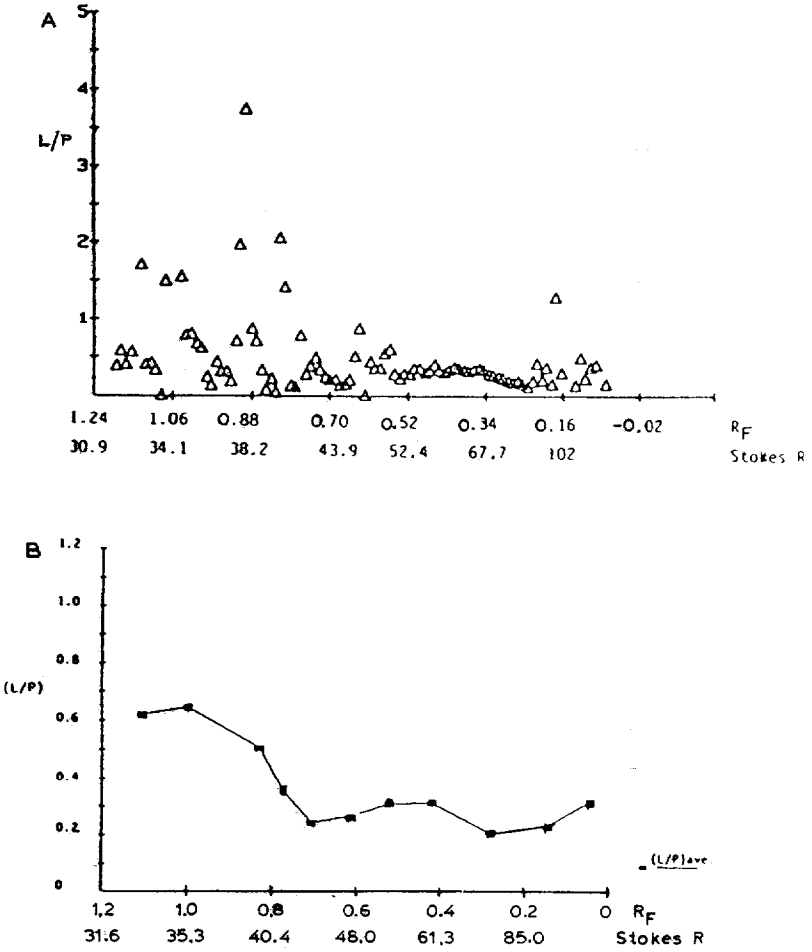

Fig. 6. (A) L/P ratios calculated from fitting 100 equi-spaced proteins to the multicomponent protein curve for sheep 1 under elevated pulmonary microvascular pressure conditions. (B) L/P ratios calculated from the 11 protein visible peaks obtained after applying the averaging technique to the data in A.

expect L/P to decrease smoothly with increasing Einstein–Stokes radius. L/P greater than unity are not expected. The eight points in Fig. 6A with L/P ratios greater than unity all occur near minima in either lymph or plasma concentration. To avoid errors associated with model predictions where little or no actual protein is present, we focused attention only on those areas in the neighborhood of visible peaks. It was assumed that for each visible peak on the electropherogram there was a protein or number of proteins of a given molecular size present. Based on this assumption, an averaging technique was used to calculate L/P ratios. This technique consists in defining a bandwidth on the multicomponent curve for protein fractions of interest, and then adding the concentration fractions obtained from the curve-fitting analysis that fall within that region. The width of the visible peak was chosen according to the following criteria.

(1) The L/P ratio for a given peak was calculated several times using as plasma and lymph protein concentrations the summation of $n+2$ protein concentration fractions located at both sides of the visible peak. N, an odd number, was varied from 1 to 9. The selection of a lower limit of three concentration fractions was based on the fact that we were restricted by the experimental accuracy of aligning the lymph and plasma peaks. Perfect superposition of both curves does not occur experimentally (see Fig. 4). The upper limit of the range is restricted by the width of the peak. Too broad a peak will include too wide a molecular size range.

(2) The presence of a plateau formed by at least two of the individual L/P ratios was taken to be indicative of the presence of a limiting L/P ratio. A plateau was defined by two consecutive L/P ratios with values within 10% of each other. The 'true' L/P ratio was considered to be the average of the L/P ratios that form that plateau.

(3) When L/P ratios were within 10% of each other but no plateau was observed, the L/P ratio was considered to be undetermined and was not included in the study.

(4) If two different plateaus were present for a given peak, the one formed by the smaller number of protein concentration fractions was considered to contain the 'true' L/P ratio. An average of the plateau values was taken to be the 'true' L/P ratio.

This averaging technique permits L/P ratios for those protein fractions that are present in very small amounts to be ignored, and allows for the fact that perfect alignment of lymph and plasma curves is not possible experimentally. Fig. 6B shows the L/P ratios for samples from sheep 1 under elevated pulmonary microvascular pressure, calculated using the averaging technique presented above.

RESULTS

Four sets of L/P ratios were calculated by combining two identical plasma samples run on different tracks on the same gel with two identical lymph samples also run on different tracks on the same gel. Both methods, the newly developed method (new method) and the method presently used in our laboratory (old method), were used. The four sets of L/P ratios were averaged for both the new and old method. The old method consisted of dividing the multicomponent protein curves into nine pre-defined protein fractions as shown in Fig. 1A and calculating the area under each fraction after subtracting the baseline drift. No effort was made to eliminate overlap from other protein fractions. Division by the total protein curve area gave the weight fraction of each component.

The same calculations were made for sheep 1 samples under baseline conditions (gel 1) and elevated microvascular pulmonary pressure (gel 2); and for sheep 2 samples under baseline conditions (gel 3) and after the injection of *E. coli* endotoxin (gel 4). Eleven protein fractions were observed on the complex plasma protein curves for sheep 1 and thirteen fractions were observed for sheep 2. Results are compared in Table II.

Fig. 7A shows the baseline and elevated pulmonary microvascular pressure L/P ratios for sheep 1 calculated using the old method and Fig. 7B shows the same

TABLE II

L/P RATIOS FOR PROTEIN FRACTIONS OBTAINED FROM THE MULTICOMPONENT PLASMA AND LYMPH CURVES CALCULATED USING THE OLD AND THE NEW METHODS

Fraction	Sheep 1		Sheep 2	
	Baseline	Microvascular pulmonary pressure	Baseline	Endotoxin
Old				
1	0.977 ± 0.098	0.761 ± 0.030	1.042 ± 0.071	0.976 ± 0.062
2	0.892 ± 0.032	0.616 ± 0.038	0.857 ± 0.022	0.762 ± 0.045
3	0.851 ± 0.018	0.474 ± 0.056	0.791 ± 0.080	0.830 ± 0.055
4	0.535 ± 0.073	0.406 ± 0.006	0.490 ± 0.060	0.576 ± 0.060
5	0.561 ± 0.012	0.166 ± 0.102	0.810 ± 0.186	0.513 ± 0.027
6	0.863 ± 0.180	0.411 ± 0.033	0.681 ± 0.052	0.692 ± 0.055
7	0.854 ± 0.099	0.370 ± 0.021	0.647 ± 0.090	0.872 ± 0.147
8	0.581 ± 0.030	0.294 ± 0.024	0.586 ± 0.087	0.725 ± 0.089
9	0.400 ± 0.027	0.181 ± 0.009	0.818 ± 0.084	0.651 ± 0.079
10	0.395 ± 0.046	0.207 ± 0.029	0.599 ± 0.076	0.537 ± 0.085
11	0.511 ± 0.095	0.340 ± 0.033	0.330 ± 0.047	0.593 ± 0.044
12	–	–	0.375 ± 0.085	0.593 ± 0.044
13	–	–	0.486 ± 0.052	0.347 ± 0.052
New				
1	0.978 ± 0.047	0.624 ± 0.079	0.942 ± 0.065	0.980 ± 0.070
2	0.906 ± 0.031	0.646 ± 0.039	0.877 ± 0.043	0.824 ± 0.057
3	0.862 ± 0.054	0.513 ± 0.019	0.722 ± 0.043	0.799 ± 0.101
4	0.689 ± 0.008	0.359 ± 0.008	0.515 ± 0.022	0.534 ± 0.036
5	0.676 ± 0.001	0.248 ± 0.020	0.671 ± 0.043	0.701 ± 0.065
6	0.658 ± 0.001	0.274 ± 0.026	0.652 ± 0.018	0.651 ± 0.007
7	0.607 ± 0.016	0.317 ± 0.018	0.669 ± 0.044	0.698 ± 0.115
8	0.679 ± 0.065	0.320 ± 0.020	0.693 ± 0.047	0.696 ± 0.073
9	0.488 ± 0.033	0.213 ± 0.005	0.713 ± 0.073	0.714 ± 0.089
10	0.555 ± 0.021	0.241 ± 0.015	0.527 ± 0.034	0.587 ± 0.069
11	0.681 ± 0.011	0.328 ± 0.028	0.353 ± 0.020	0.503 ± 0.027
12	–	–	0.531 ± 0.019	0.570 ± 0.021
13	–	–	0.410 ± 0.133	0.497

L/P curves calculated using the new method. Fig. 8A shows the baseline and endotoxin L/P ratios for sheep 2 samples calculated using the old method and Fig. 8B shows the same L/P curves calculated using the new method.

If the new method of analysis eliminates the problem of curve overlap by decomposing the multicomponent electropherogram into single protein fraction curves, L/P ratios calculated using the new method would be expected to be more accurate than the ones calculated using the old method. The old method shows more scatter in L/P values. Furthermore, the curves obtained by the new method are smoother than the ones obtained with the old method. This corresponds to what one might expect from theory. When the new method is used, the L/P ratios show a distinct flat region between the molecular sizes of 50 and 60 Å.

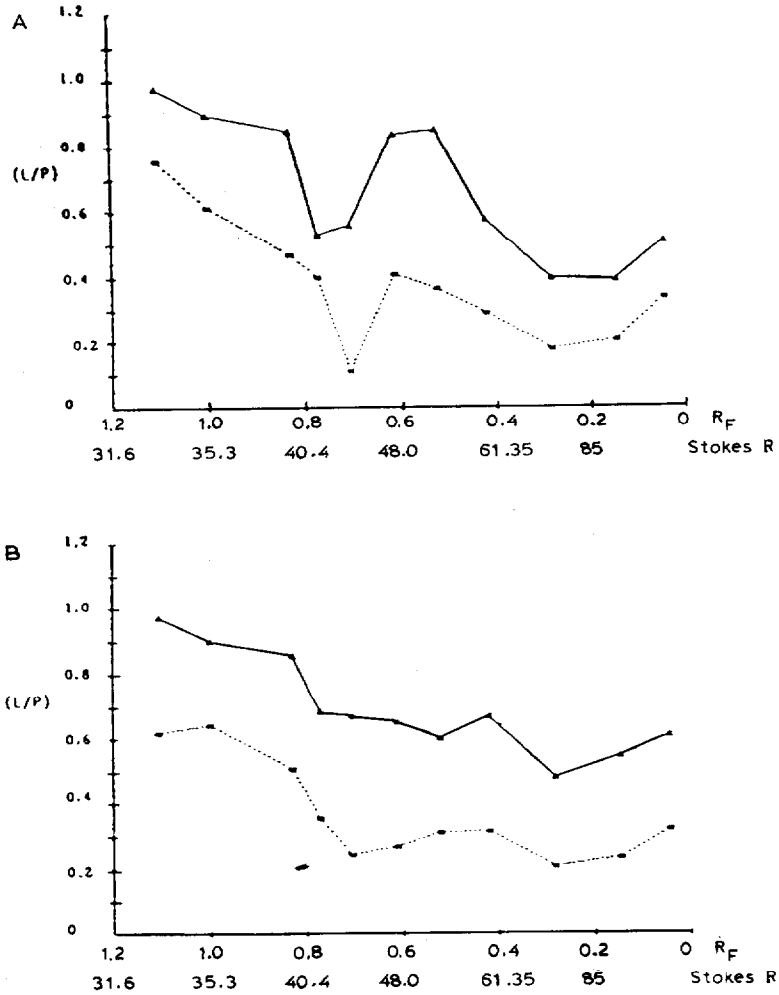

Fig. 7. Average L/P ratios for sheep 1 under baseline (solid line) and increased pulmonary microvascular pressure (dashed line) conditions calculated for the eleven visible protein electropherogram peaks using (A) the old method and (B) the new method.

The L/P ratios calculated using the old method fluctuate greatly, but for the most part appear to provide reasonable estimates of actual L/P. The baseline L/P ratios for sheep 1 and 2 are expected to be similar since the experimental conditions are the same. Notice that the L/P ratios for sheep 1 and 2 calculated using the new method are similar (Figs. 7B and 8B). On the other hand, the L/P ratios calculated using the old method for the same sheep under the same conditions do not agree as well (Figs. 7A and 8A).

Figs. 8A and B are excellent examples of the improved accuracy of the L/P ratios obtained using the new method. Since *E. coli* endotoxin increases the permeability of the membrane, an increase from baseline of the L/P ratios is expected for all the proteins, especially for the larger ones. Fig. 8A shows so much scatter between baseline and endotoxin L/P ratio values that it is almost impos-

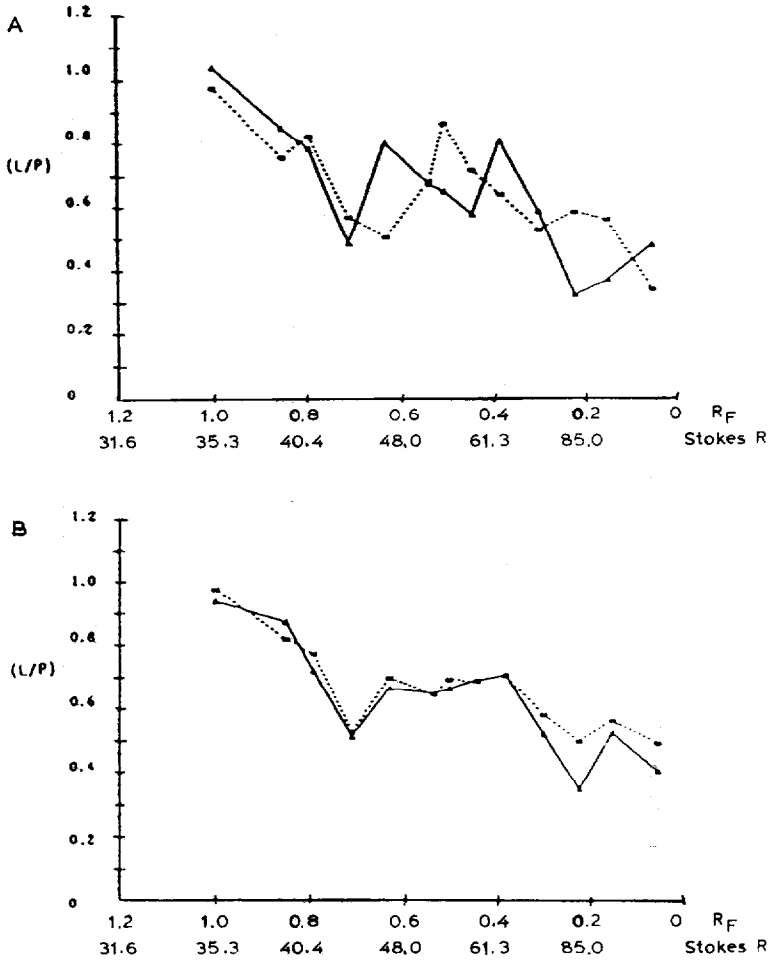

Fig. 8. Average L/P ratios for sheep 2 under baseline (solid line) and after *E. coli* endotoxin injection (dashed line) conditions calculated for the thirteen visible protein electropherogram peaks using (A) the old method and (B) the new method.

sible to see the effect of endotoxin on the L/P ratios. On the other hand, Fig. 8B shows a clear distinction between baseline and endotoxin L/P ratios for large proteins.

DISCUSSION

The linear relationship between the logarithm of the Einstein–Stokes radius and the logarithm of the electrophoretic mobility R_F was obtained using proteins which have exclusively globular molecular shapes. Non-globular proteins behave differently in gel electrophoresis. When proteins with elongated molecular shapes are electrophoresed, and their Einstein–Stokes radius, R, calculated from their migration distance using the linear relationship for globular proteins, they show an apparent size which is smaller than their actual size. This phenomenon can be explained by the electrophoretic alignment [13]. In gel electrophoresis, the

molecules are aligned with the electrical field causing them to be pulled into the gel pores by their smaller molecular axis, giving the elongated molecules an apparent radius smaller than their Einstein–Stokes radius. For globular molecules the effect of electrophoretic alignment is minimized.

In the present analysis of protein fractions in gel electrophoresis, it was assumed that only globular proteins were present. This is not entirely true since plasma and lymph contain non-globular proteins. The effect of elongated proteins on the L/P values obtained by assuming that only globular proteins are present in the lymph and plasma samples shows an unexpected decrease in the value of L/P. This is particularly noticable at Einstein–Stokes radii near 45 Å. This apparent decrease in L/P is due to the fact that the equivalent size of elongated proteins as measured by gel electrophoresis is smaller than their Einstein–Stokes radii.

Another error associated with the behavior of elongated proteins on gel electrophoresis is related to the shapes of their electrophoresis curves. Since globular proteins do not differ much from one another in their molecular shapes, their electrophoresis curves should be only a function of the gel concentration as mentioned above. But acrylamide electrophoresis curves for elongated proteins depend also on their molecular shape, and consequently they cannot be simply determined from electropherograms of pure globular proteins. Our method of analysis does not correct for deviations caused by non-spherical protein shape. To correct for the effects of non-spherical shape, these proteins would have to be identified, purified and used as their own reference.

The noisy L/P ratios obtained from the 100 protein fractions (Fig. 6A) were quite unexpected. Since the overlap of the multicomponent curves was eliminated by the curve-fitting technique, one would expect accurate L/P ratios for the protein fractions obtained from the curve fitting. It was found that the noisy L/P ratios were due to two main factors: (1) small amounts of protein present in the valleys of the multicomponent electropherograms; (2) experimental variability in gel acrylamide concentration giving a misalignment of some of the protein peaks in the plasma and lymph electropherograms. This erroneous alignment will result in inaccurate L/P ratios since the concentration of the mth plasma protein in sample will not correspond exactly to the concentration of the mth lymph protein fraction. Consequently, the averaging technique described here significantly reduced these two problems.

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