CHROM. 3986

THE EFFECTS OF SOME VARIABLES ON PROTEIN SEPARATION BY POLYACRYLAMIDE GEL ELECTROPHORESIS

C. J. BRACKENRIDGE AND H. S. BACHELARD
Department of Biochemistry, Institute of Psychiatry (British Postgraduate Medical Federation, University of London), De Crespigny Park, London S.E. 5 (Great Britain)

(Received January 24th, 1969)

SUMMARY

The effects of several variables on the migration and resolution of human serum proteins in polyacrylamide gel was examined by means of the cylindrical electrophoretic technique. It was found that photopolymerization of gels should proceed for ample time, one hour under present conditions, prior to sample application. When it is desired to compare several protein solutions and sucrose is added to confer density, their final sample volumes should be identical and the final sucrose concentration should be at least 5% (0.15 *M*).

The migration of proteins was retarded as the proportion of acrylamide or its comonomer, methylenebisacrylamide, increased in the gel. Optimal resolution of fractions was observed at a total amide concentration of about 7%. Relative to albumin, proteins of slower mobility, such as α_2-globulins, show maximum separation at this gel concentration. It was possible to relate the proportions of acrylamide and cross-linking agent conducive to the best fractionation in an inverse linear manner.

INTRODUCTION

The method of DAVIS[1] applied the theoretical considerations of ORNSTEIN[2] to the electrophoretic analysis of serum proteins (about 200 μg in 3 to 4 μl) on three layers of acrylamide gel. CLARKE[3] replaced the two upper sections used to concentrate the proteins by a dense sucrose solution of the sample. A similar modification had been independently reported by BROOME[4], who introduced a buffered suspension of Sephadex G-200 gel in sucrose to combat convection in the medium. Such a mixture of lower conductivity than the electrode buffer was shown[5] to be as efficient a means of sharpening the protein zones as a discontinuous buffer system.

In adapting these simplifications to a procedure suitable for the separation of proteins from mammalian cerebral cortex, information appeared to be deficient on three important experimental variables.

(1) To catalyse gel formation it was considered wise to avoid the use of persul-

phate ions in view of their adverse oxidative properties[6,7] towards enzymes. Riboflavin-sensitized photopolymerization commended itself as a more flexible alternative, particularly when combined with an accelerator. No study appears to have been made of the effect of irradiation time on the electrophoretic pattern.

(2) Low acrylamide concentrations produce gels of large pore size and are therefore suitable for fractionating high molecular weight substances. Conversely, high gel concentrations are useful for peptide fractionation. While rough guides[1,2] are available for choosing an acrylamide content appropriate to the size of the macromolecules requiring analysis, virtually no information exists on the secondary problem of selecting the amount of comonomer for cross-linkages producing optimal mechanical properties and best resolution of protein bands and whether these coincide.

(3) Brain tissue extracts are often prepared in media containing materials such as sucrose or buffers which may influence the migration and resolution of proteins during electrophoresis. The effects of sample composition and volume therefore needed to be tested.

Human blood serum is a readily available source of a complex, yet well-characterized, system of proteins possessing a wide range of mobilities. The present report describes the effects of variation in time of photopolymerization, extent of cross-linkage, and composition of the sample medium on the patterns obtained by acrylamide gel electrophoresis in order to determine optimal conditions.

MATERIALS AND METHOD

Preparation of gels

Acrylamide and the comonomer N,N'-methylenebisacrylamide (Bis), products of Kodak Ltd., Kirkby, England, were recrystallized according to LOENING[8]. Specified concentrations of each were made up in electrode buffer, 0.05 *M* glycine-Tris, pH 8.5, prepared from reagents of analytical grade. Riboflavin-5'-phosphate (Koch-Light Laboratories Ltd., Colnbrook, England) and N,N,N',N'-tetramethylethylenediamine (Kodak Ltd.) were added to make final concentrations of 0.6 mg/100 ml and 40 μl/100 ml, respectively. Aliquots of 1.2 ml were added to eight stoppered tubes of internal diameter 0.5 cm and length 7.5 cm. Solutions were photopolymerized for a given time using a Photopol fluorescent lamp with two 6 W daylight tubes, all apparatus being from the Shandon Scientific Co. Ltd., London.

Electrophoresis

Unless otherwise indicated, protein solutions were prepared to contain sucrose (10%, w/v) and electrode buffer (10%, v/v). Volumes up to 300 μl containing up to 200 μg of protein were applied to the gels. In a typical experiment eight solutions of identical protein concentration were submitted to electrophoresis at 150 V and 10–12 mA for 50 min at room temperature. Buffer in both compartments was changed after each run. After ejection from their tubes, gels were stained for protein in a 1% solution of Naphthalene Black (G. T. Gurr Ltd., London) for 30 min in methanol–water–glycerol–acetic acid (50:50:20:1), as described by TOMBS[9]. Excess dye was removed by rinsing with the same solvent mixture; the procedure was hastened by rotation on a wheel at 30 rev./min.

Human serum

Serum was separated from clotted blood by centrifugation and frozen until required. Only samples from a single source were used in comparative studies and in those examining the effects of a variable on gel electrophoretic patterns.

RESULTS AND DISCUSSION

Effect of photopolymerization time

The distances moved by five major proteins of human serum in gels which had been irradiated for various times are shown in Fig. 1. The length of the gel cylinder and the distance traversed by albumin both increased during the first 20 min and did not alter thereafter. In the α_2-globulin region, for which a terminology has been proposed by CLARKE[3], the behaviour was more complex. The order of migration of two adjacent bands, transferrin and 1-α_2, was dependent on the time of photopolymerization. At times less than 30 min the 1-α_2 band preceded transferrin; at longer times the positions were reversed. Like 1-α_2, the 2-α_2 band showed an appreciable decrease in migration with time, but the rate of migration of the 10-α_2 band was independent of the time.

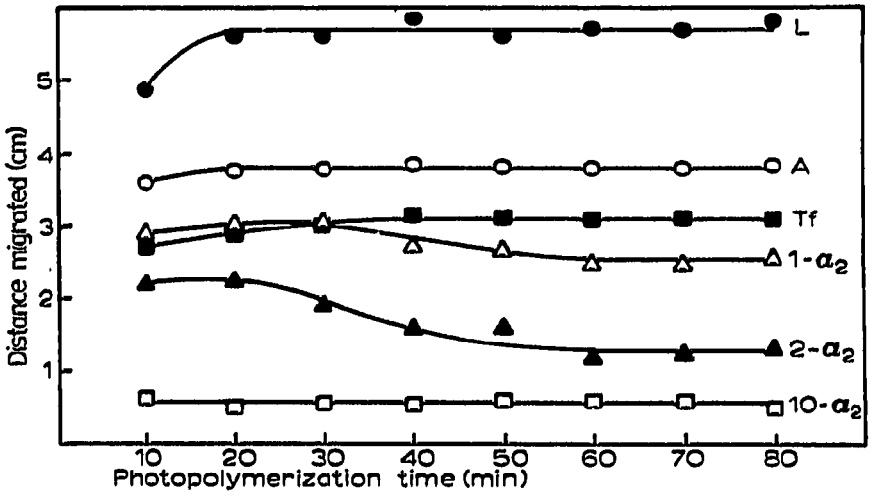

Fig. 1. Effect of irradiation time for photopolymerization of 7% acrylamide gels on length of gel (L), and migration of albumin (A), transferrin (Tf) and globulins[3] (1-α_2), (2-α_2) and (10-α_2).

It is possible that, as photopolymerization proceeds, changes in porosity affect the mobility of proteins to an extent dependent on their molecular dimensions. Thus narrowing of the interstices readily explains the behaviour of the 1-α_2 and 2-α_2 fractions; the ratios of their migration distances to the gel length fell by 25% and 50%, respectively, from 10 to 60 min. Transferrin, which appears to act differently, retained a constant ratio of 0.54 $\pm$ 0.03 after 20 min, as also did albumin (0.67 $\pm$ 0.02) and the 10-α_2 band (0.10 $\pm$ 0.01). The migration distance of all bands did not change after 60 min, so this period was adopted in all subsequent experiments. The need for polymerization to proceed to completion seems to apply irrespective of how it is brought about. GANDINI AND HYDÉN[10] obtained different electrophoretic patterns at various stages of gelation by changing the proportion between tetramethyl-

ethylenediamine and ammonium persulphate. They found the less the polymerization was advanced, the more pronounced was the swelling of the gel.

Effect of gel composition on migration and resolution

The distances migrated by albumin (A) and two other proteins in human serum were used to assess the influence of the cross-linking agent on distance of migration. Termed F (fast) and S (slow), they have been tentatively identified as the 2-β (ceruloplasmin) and 5-α_2 bands, respectively, of CLARKE[3]. The 2-β band migrated at about two-thirds and the 5-α_2 band at about one-third the rate of albumin: the three fractions therefore cover a broad range of mobilities. The length of the gel cylinder and the total number of distinguishable bands in the pattern were also determined over a wide range of total gel concentration. Fig. 2 shows the results obtained with

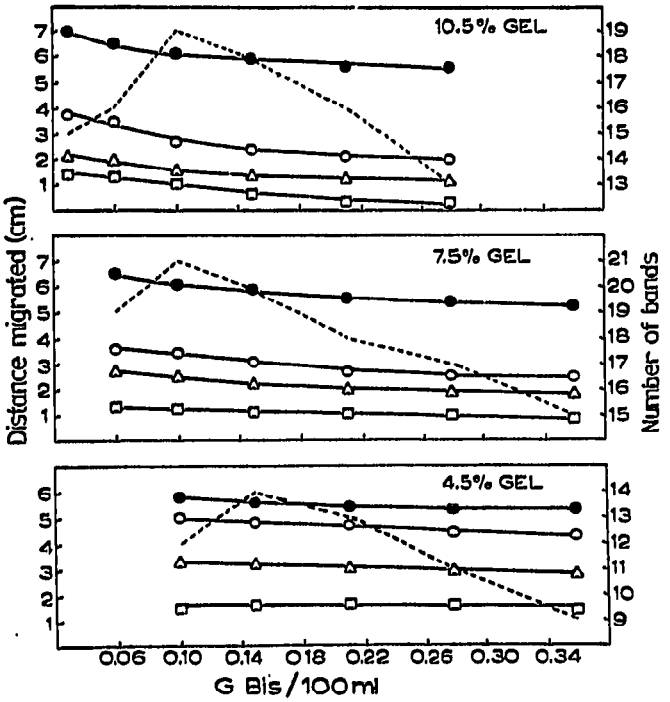

Fig. 2. Effect of methylenebisacrylamide concentration in gels of total amide concentration 4.5%, 7.5% and 10.5% on length of gel (L), and migration of protein bands A, F, and S after electrophoresis for 30 min. For details see text. Broken lines represent the number of distinguishable fractions (right hand side ordinate).

4.5%, 7.5% and 10.5% gels. In 4.5% gels the proteins migrated and the gel length decreased almost linearly with increasing proportions of Bis in the polymerization mixture; at higher gel concentrations, the same trend was observed but with a more marked departure from linearity especially at the low Bis concentrations. The results therefore agree with those of JONGKIND, WISSE AND BLOEMENDAL[11], who found that variation in the amounts of comonomer greatly affected the penetration of α-crystallin into the gel. Using 0.2% Bis, a 'small-pore' gel was obtained, whereas addition of only 0.05% Bis resulted in a 'large-pore' gel.

The number of bands discernible was higher in the 7.5% gel than in the others, and was strongly dependent on the extent of cross-linkage. The concentration of Bis

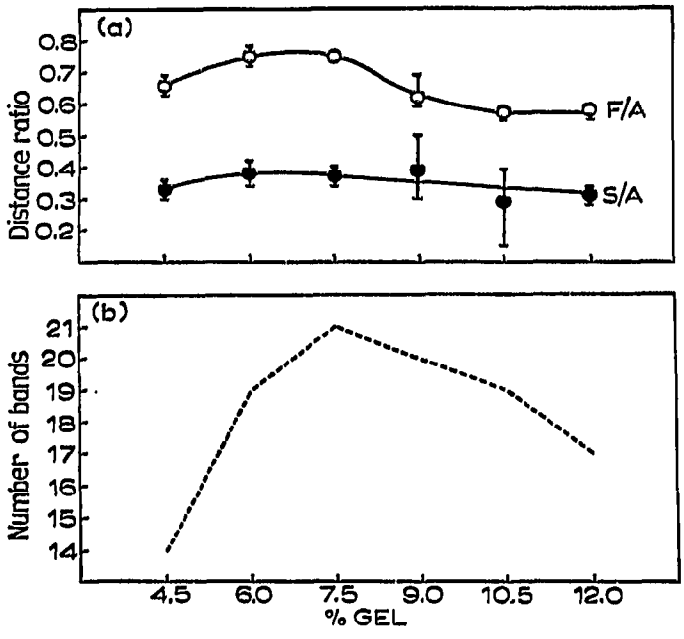

Fig. 3. (a) Variation of protein migration ratios F/A (upper) and S/A (lower) with total amide content, showing variation of values over a range of methylenebisacrylamide concentrations. For details see text. (b) Variation of highest number of distinguishable fractions with total amide content over the same range of methylenebisacrylamide concentrations.

required for optimal resolution decreased with increasing total amide content; this inverse relation has been noted by DAVIS[1] to give the most desirable polymer properties when preparing gels.

While there was clearly a decrease in protein migration with increase in cross-linkage, it was necessary to determine whether this applied to the same extent to these bands. In Fig. 3a, the migration ratios F/A and S/A are plotted as a function of gel concentration. Each value represents the mean of the ratios measured at a particular gel concentration for a range of Bis concentrations which were 0.06 to 0.36 g/100 ml (4.5 to 9.0% gels) and 0.03 to 0.28 g/100 ml (10.5 and 12.0% gels). For a given gel concentration, the differences between ratios was within experimental error except at 9.0% and, for S/A only, at 10.5%. In these two cases, there was a systematic variation over the range shown: it was greater for S/A than for F/A, and the ratios dropped with rise in Bis levels. The migration rates of the slow band (S) and, to a lesser extent, the fast band (F) are therefore strongly dependent on cross-linkage over a small range of gel concentration: this is presumably due to a critical change in pore size. A maximum in both curves can be seen close to 7% gel concentration. The fact that this also corresponds to the region of maximum resolution (Fig. 3b) can probably be related to the profuse number of proteins occurring in the α_2-globulin area[3]. These become more readily distinguishable as their migration distances increase relative to the protein-sparse albumin area. It is of interest that RAYMOND AND NAKAMICHI[12] found gel concentrations in the range 5 to 10% to be the most useful for serum protein separations on slabs of acrylamide gel.

In Fig. 4a the decrease in migration with increasing gel concentration at a constant Bis concentration of 0.15 g/100 ml is illustrated. The overall effect was similar to that seen in Fig. 2, except that the gel expanded very slightly with in-

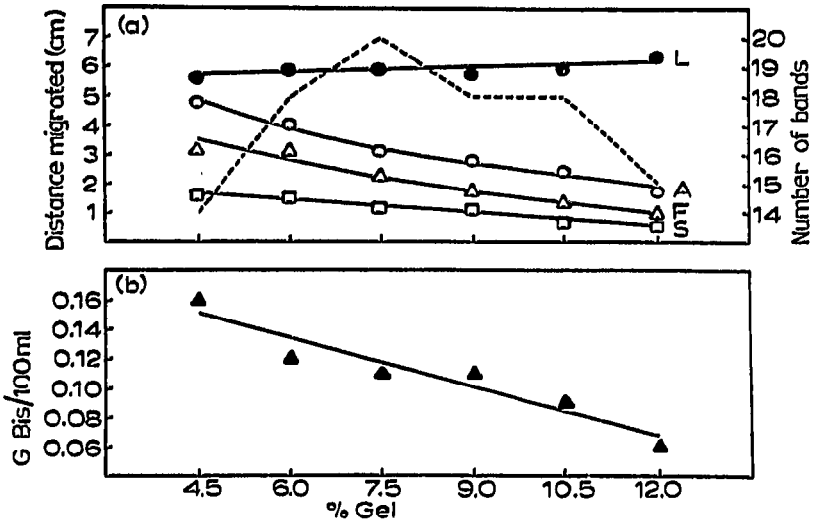

Fig. 4. (a) Effect of acrylamide concentration in gels of constant methylenebisacrylamide content (0.15 g/100 ml) on length of gel (L), and migration of protein bands A, F, and S. For details see text. Broken lines represent the number of distinguishable fractions (right hand side ordinate). (b) Relation between interpolated methylenebisacrylamide concentration producing optimal protein resolution and total amide content of gels.

creasing total amide content in contrast to the contraction observed with increasing Bis concentrations. The inverse relation between gel concentration and absolute migration is in agreement with larger-scale work carried out on vertical gel slabs[12–14]. The number of discernible fractions (Fig. 4a) was greatest in the 7.5% gel as in Fig. 3b. When the Bis concentration producing the highest resolution was estimated to the nearest 0.01 g/100 ml, from data such as in Fig. 2, and plotted against the appropriate gel concentration, the result is seen in Fig. 4b. This permits the calculation of the optimal cross-linkage for a given gel percentage; the least-squares regression equation is given by

$$B = 0.201 - 0.0112\,G,$$

where B is the Bis concentration in % and G is the total gel concentration in %.

Fig. 5 shows a typical separation of proteins on a 7% gel (6.88% acrylamide and 0.12% Bis) with 24 discernible bands. The number seen depends not only on experimental conditions but on the nature of the sample; genetic variants of some proteins (particularly the haptoglobins) give rise to a different number of bands[15]. DAVIS[1] and PASTEWKA, NESS AND PEACOCK[15] found 20 to 30 fractions on their 5% gels.

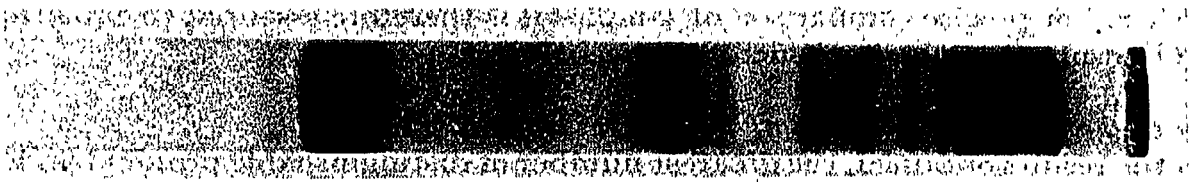

Fig. 5. Electrophoretic pattern of proteins on 7% polyacrylamide gel (4 μl of human serum containing approx. 300 μg of protein).

Effect of sample composition on migration

Sucrose solutions of protein, on which the electrode buffer can be layered, are a useful means of minimizing the sample volume to facilitate band-sharpening. Such mixtures also arise directly from tissue homogenization and sucrose density-gradient centrifugations. In these cases it is common practice to compare solutions containing similar amounts of protein. This often means that the weight and volume of sucrose differ from sample to sample, hence it is relevant to examine whether the composition of the sample medium influences migration.

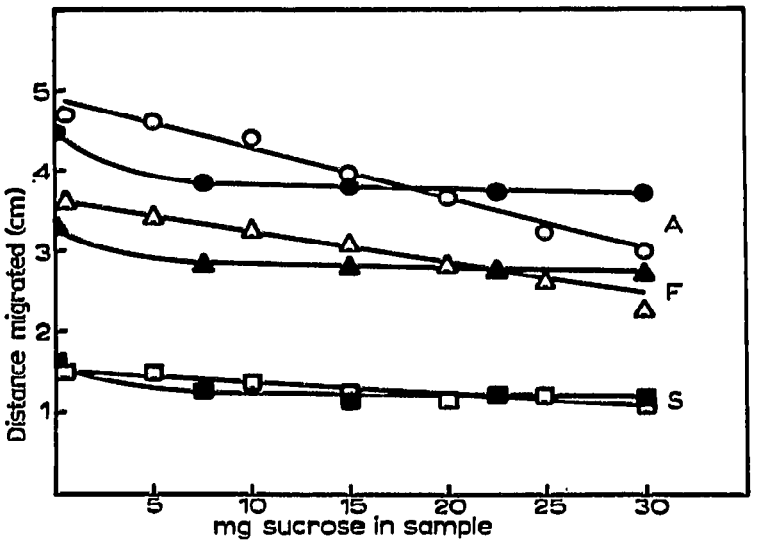

Fig. 6. Effect of variation in weight of sucrose in protein samples on migration of bands A, F, and S in gels of 7% total amide content. Open symbols refer to different volumes (5–300 μl) of constant sucrose concentration (10%); closed symbols refer to constant volumes (150 μl) of different sucrose concentrations (0–20%). Sample protein content was constant throughout.

Fig. 6 reveals the effects on bands A, F and S caused by increasing sample volumes of sucrose solutions of constant composition and by constant sample volumes of increasingly concentrated sucrose solutions. The gel composition was 6.9 g acrylamide/100 ml and 0.1 g Bis/100 ml. It can be seen that the rate of protein migration decreased linearly with increasing sample volume, whereas sucrose concentrations greater than 5% (7.5 mg in a constant sample volume of 150 μl) did not influence the migration appreciably, providing the volumes remained constant. Migration ratios were unaltered throughout: the observed values for F/A of 0.77 $\pm$ 0.05 and S/A of 0.34 $\pm$ 0.04 at constant sucrose concentration coincide with ratios of 0.74 $\pm$ 0.01 and 0.33 $\pm$ 0.04 when the volume is fixed. The addition of Sephadex G-200 gel to reduce convection[4] resulted in a definite retardation of migration and was not studied further. For precise comparison of samples it is therefore essential to equalize volumes and ensure that the final sucrose concentration exceeds 5%.

Application to the optimal resolution of protein mixtures

From the results obtained, the greatest number of distinguishable protein bands in human serum is found in 7% acrylamide gels containing about 0.12% methylene-bisacrylamide. This is probably because the numerous α_2-globulins are best resolved

under these conditions, and is likely to be of general relevance to those systems which contain proteins migrating between one-third and two-thirds as fast as human serum albumin. In other cases the relation shown in Fig. 4b should enable prediction of the proportion of cross-linking agent appropriate to a given acrylamide gel content for convenient mechanical properties and good resolution of components.

It is necessary for gel polymerization to continue to completion, otherwise some fractions may migrate in an unusual manner. Bands are sharpened if the sample is applied to the gel in a medium of low conductivity[5] and suitable density. The rate of migration of proteins has been shown to be independent of the final sucrose concentration of the sample, provided it is above 5% (0.15 *M*), but to depend upon the sample volume. This is of relevance to comparisons of protein constituents of different tissue extracts when the sample volume may be chosen according to the protein concentration. The results presented here refer to sucrose but other sample medium constituents may also influence the migration rates. It seems advisable, therefore, when comparative electrophoresis of different protein samples is to be studied, that care should be taken to ensure uniformity of sample volume and non-protein constituents.

ACKNOWLEDGEMENTS

Appreciation is expressed to the Medical Research Council for financial support. This study was carried out during the tenure by C.J.B. of a C. J. Martin Fellowship provided by the National Health and Medical Research Council of Australia.

REFERENCES

1 B. J. DAVIS, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404.
2 L. ORNSTEIN, *Ann. N.Y. Acad. Sci.*, 121 (1964) 321.
3 J. T. CLARKE, *Ann. N.Y. Acad. Sci.*, 121 (1964) 428.
4 J. BROOME, *Nature*, 199 (1963) 179.
5 S. HJERTÉN, S. JERSTEDT AND A. TISELIUS, *Anal. Biochem.*, 11 (1965) 219.
6 J. M. BREWER, *Science*, 156 (1967) 256.
7 W. M. MITCHELL, *Biochim. Biophys. Acta*, 147 (1967) 171.
8 U. E. LOENING, *Biochem. J.*, 102 (1967) 251.
9 M. P. TOMBS, *Anal. Biochem.*, 13 (1965) 121.
10 S. GANDINI AND H. HYDÉN, *J. Chromatog.*, 32 (1968) 715.
11 J. F. JONGKIND, J. H. WISSE AND H. BLOEMENDAL, *Protides of the Biological Fluids (10th Colloquium, Bruges, 1962)*, 10 (1963) 77.
12 S. RAYMOND AND M. NAKAMICHI, *Anal. Biochem.*, 7 (1964) 225.
13 J. ŻWAAN, *Anal. Biochem.*, 21 (1967) 155.
14 R. HOLMES, *Biochim. Biophys. Acta*, 133 (1967) 174.
15 J. V. PASTEWKA, A. T. NESS AND A. C. PEACOCK, *Clin. Chim. Acta*, 14 (1966) 219.