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A SIMPLE SPECTROPHOTOMETRIC SCANNING PROCEDURE FOR QUANTITATIVE ESTIMATION OF PROTEIN IN POLYACRYLAMIDE GELS

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SUMMARY

A simple and rapid spectrophotometric procedure for the scanning of polyacrylamide gels in ultraviolet light is described.

It is shown that the absorption of the albumin band in acrylamide gel is proportional to the quantity of albumin subjected to electrophoresis and that the procedure can be applied to the study of serum and tissue protein separations in polyacrylamide gels. Some of the advantages of this method over other currently used methods are indicated.

INTRODUCTION

In using the polyacrylamide gel electrophoresis method of ORNSTEIN[1] and DAVIS[2] for metabolic studies of proteins two problems are encountered. The first is the quantitative estimation of protein in the individual bands, and the second is the accurate measurement of radioactivity in the protein discs in acrylamide gels.

A number of methods are available for radioactivity measurements in protein bands[3–5]. However, the methods currently used for estimation of protein in acrylamide gels, namely, the densitometric scanning of stained gels or their photographic negatives, or the extraction of the dye bound to protein discs with solvents[6,7], are in general tedious and time consuming. A method of direct scanning of acrylamide gels for the study of cytochrome c has been reported[8] taking advantage of the colour of cytochrome c for its detection and quantitation. A short note describing a scanning device for polyacrylamide gels has appeared recently[9].

In the present communication a simple procedure for the scanning of acrylamide gels in U.V. light is described. The scanning device can be connected to a spectrophotometer in place of the standard sample changer. A quantitative measurement of albumin as a test substance and the applicability of the method to serum and tissue protein separations are also reported.

METHODS

Apparatus

A standard Zeiss Spectrophotometer (PMQ II) was modified for the scanning

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of acrylamide gels. The sample changer and the detector housing were replaced by the chromatogram scanning attachment available from Zeiss. The schematic diagram of the scanner and the gel holder is shown in Fig. 1. The gel holder was constructed from a metal plate, 32 × 168 × 3.5 mm. A slot with bevelled edges, 3 mm wide facing the monochromator and 9 mm towards the photomultiplier, was machined lengthwise into this plate. The quartz tube with the gel fits into the slot and is held in place by Scotch tape. The gel holder is securely clamped to the sliding shelf of the chromatogram scanner in such a fashion that it can be easily removed yet properly aligned when reinserted. The sliding shelf, which can travel in the horizontal plane, is in turn driven by a constant-speed motor.

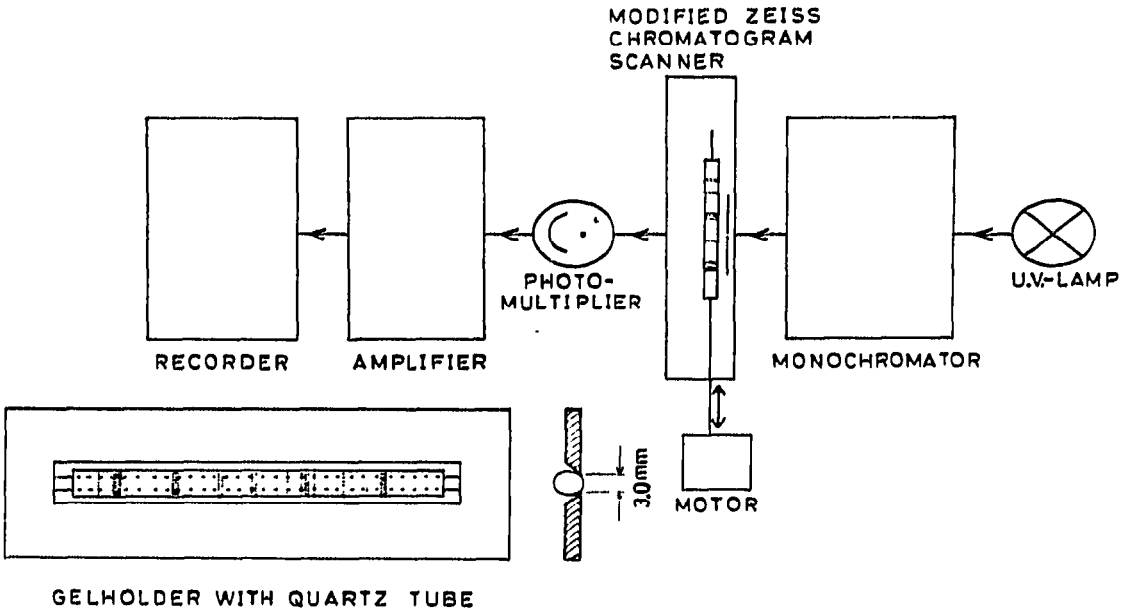

Fig. 1. A schematic diagram of the scanner and the gel holder.

In order to be able to use the maximum intensity of light per unit area from the monochromator which thereby permits the use of a smaller band width of the monochromatic light, the chromatogram scanning assembly had to be modified slightly. The extension tube with a cylindrical lens was removed and the chromatogram scanner placed directly against the side of the monochromator housing. A vertical slit 50 μm wide was placed in between the monochromator and the gel holder. During scanning, the gel is transported across the slit and the optical density recorded by a Vitatron (Model-UR 400) recorder connected to the indicating unit of the spectrophotometer. Due to the disparity between the output of the indicating unit and the input of the recorder, the signal from the indicating unit was amplified 10-fold before it was fed into the recorder.

It should be noted that the device described above is inexpensive and of simple design. Moreover, the adaption of the spectrophotometer for the scanning of gels does not impair the general operation of the instrument. The changeover can be accomplished in about 5 min. In addition, the arrangement of the gel holder is such that the gel tubes can be changed in less than a minute, allowing the scanning of several gels without losing time.

Electrophoresis

Polyacrylamide gel electrophoresis was carried out in quartz tubes 7 cm long with an internal diameter of 5 mm. The quartz tubes are readily available (Kebo, Stockholm) and are not expensive. Optically the quartz glass was satisfactory for the present work; the absorption of these tubes (empty) at 280 nm was 0.065, and at 230 nm it was 0.565. A batch of 6 tubes was cut out from a long length of tubing, and the variation in absorption from tube to tube was negligible.

Electrophoresis of crystalline bovine serum albumin and calf serum was performed according to the method of ORNSTEIN[1] and DAVIS[2]. For electrophoresis of tissue homogenates the above technique was slightly modified. Brain and liver samples were homogenized in approximately 5 volumes of 0.25 *M* sucrose solution and centrifuged at 105000 × *g* for one hour. The clear supernatants were used for electrophoresis. The sample was placed over the spacer gel and was mixed with dry Sephadex G-100, to avoid convective losses of protein during electrophoresis.

The pH of the separation gel was 8.9. Serum and albumin samples were run in 7.5 % acrylamide concentration. For electrophoresis of brain and liver protein 11.2 % polyacrylamide was used. The samples were run for 2–3 h at + 4° when the electrophoretic front had migrated 30–40 mm from the beginning of the separation gel.

After scanning at 280 nm the gels were stained in 1 % amido black in 7.5 % acetic acid for 24 h and the excess dye was removed by repeated washing in 7.5 % acetic acid.

The densitometric tracings of the photographic negatives of stained gels were carried out using a Schnell Photometer II (Zeiss, Jena), connected to a Vitatron recorder. Photographs of stained gels were taken with an expanded Hasselblad camera, Kodak verichrome Pan (ASA-125) film, a lens to gel distance of 12 cm, diaphragm f 22, and 1–2 sec exposure time.

Spectrophotometric procedure

At the end of the electrophoretic run the quartz tubes were cleaned carefully with a lens paper and placed in the gel holder which was mounted on the sliding shelf of the scanning assembly. At a wavelength of 280 nm the base line was fixed at 100 % transmission in that part of the gel ahead of the electrophoretic front, and the optical density over the length of the gel was recorded. The full scale of the recorder chart can be adjusted to read from 0.2 to 2.0 optical density units, thereby permitting the recording of protein bands with a wide range of concentration.

After scanning at 280 nm the gels were stained and destained as described under METHODS. The time necessary for scanning a gel was about 2–3 min from the termination of electrophoresis. The diffusion of protein bands during this time could certainly be expected to affect the resolution, especially of closely spaced bands. Gels stained and fixed immediately after electrophoresis and after 2–3 min did indicate diffusion of protein discs to some extent, although there were no marked alterations in the protein patterns.

The stained gels were introduced into quartz tubes of slightly larger diameter and scanned as described above at 550 nm to compare the results obtained in the two types of recording. In some cases densitometry of the photographic negatives of stained gels was also performed.

RESULTS AND DISCUSSION

Electrophoresis of various concentrations of crystalline bovine serum albumin in 7.5 % acrylamide gel was performed as described. Upon termination of the electrophoretic run the gels were scanned at 280 nm. The area under the peaks was calculated and plotted against albumin concentration. The results presented in Fig. 2 indicate a linear relationship between albumin concentration and the corresponding peak area over the range of albumin concentration (1–50 μg) tested.

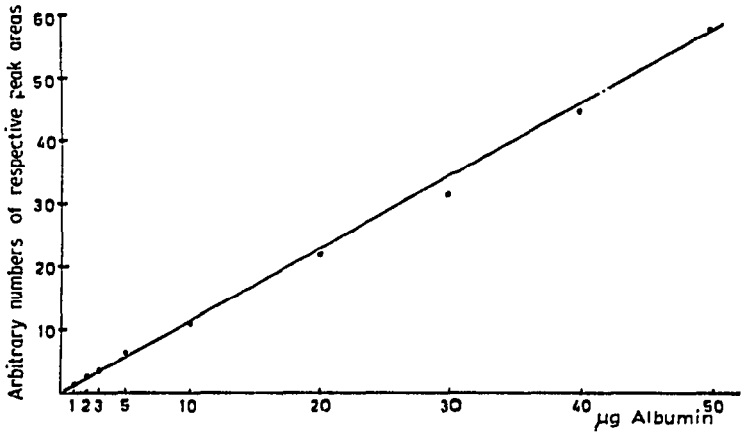

Fig. 2. Absorption of crystalline serum albumin in 7.5% acrylamide gel.

All the scannings performed in the ultraviolet range were at 280 nm. It is known that proteins and polypeptides absorb at an increasing rate below 240 nm. Ideally a choice of 230 nm or a lower wavelength should result in considerably enhanced sensitivity. The small absorption peak of a solution of crystalline albumin (pH 8.5) at 280 nm was only about 10 % of the absorption at 230 nm. A wavelength of 280 nm, however, was selected due to the fact that the self-absorption of polyacrylamide gels increases markedly below 250 nm, and the gels are completely opaque at 230 nm. Recrystallization of acrylamide and bisacrylamide as suggested by LOENING[10] did not alter the absorption characteristics of these gels below 250 nm. As a rule, all the gels used in this study were polymerized from recrystallized monomers. The quartz tubes used in this study also showed considerable absorption at 230 nm. However, the opacity of these gels at this wavelength prohibits the scanning of acrylamide gels even with quartz glass of the best quality.

The applicability of the method to the study of serum and tissue protein separations was verified by subjecting these samples to electrophoresis under similar conditions. Electrophoresis of 1 μl of calf serum was performed, and the gels were scanned at 280 nm. A typical scan of such a gel is shown in Fig. 3A. It is apparent that most of the components of serum can be satisfactorily resolved and can be quantitated using albumin as the standard. It seemed interesting to check how the scanning of gels stained with amido black at 550 nm, using the above device, would compare with the one at 280 nm. A scan of the same gel as in Fig. 3A after staining is presented in Fig. 3B. It can be seen that in general, scanning at either 280 or 550 nm results in very similar resolutions. A closer examination of these two tracings, however, indicates that

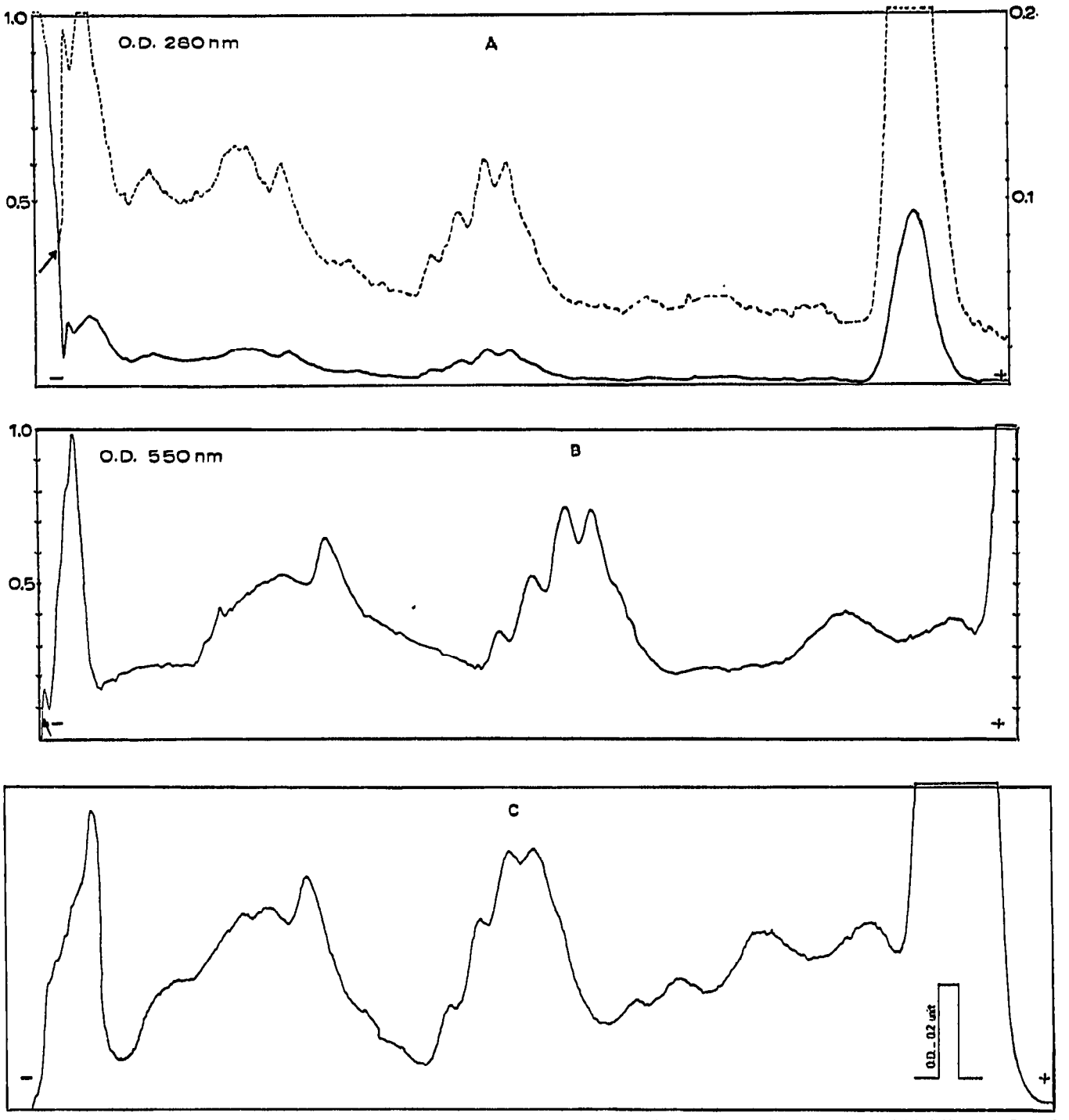

Fig. 3. Scans of the separation of calf serum protein in 7.5% polyacrylamide gel. (A) The unfixed and unstained gel was scanned at 280 nm immediately after electrophoresis. The continuous line represents a scan with the full length of the recorder chart set at 1.0 optical density unit; the broken line tracing was carried out with the recorder chart set at 0.2 optical density unit. The arrow indicates the beginning of the separation gel. The large anodal peak corresponds to albumin. The recorder speed was 10 × that of the gel. (B) The same gel was stained with amido black and scanned at 550 nm with the recorder chart set at 1.0 optical density unit. The arrow indicates the beginning of the separation gel. The albumin peak is only partially included in the tracing. The ratio of recorder speed to that of the gel was the same as in (A). (C) The same stained gel was photographed and the densitometric tracing of the negative was perfomed as described in the text. Chart calibration is shown in the figure. The recorder speed was 10 × that of the gel.

the relative proportions of some of the protein discs in the same gel are changed. The differences in the binding capacity of amido black with different proteins could contribute to such a discrepancy. To compare these results further with the widely used technique of densitometry of photographic negatives of stained gels, a densitometric tracing of the same gel as in Figs. 3A and 3B is shown in Fig. 3C. A comparison of Figs. 3B and 3C also indicates differences in the relative proportions of some components. Investigation of the exact quantitative relationship of different protein fractions in a sample as affected by scanning of acrylamide gels at 280 or 550 nm is in progress. Some of the differences in resolution in Figs. 3B and 3C could probably be attributed to the difference in scanning sensitivity used.

A U.V.-scan of brain stem homogenate, subjected to electrophoresis in 11.2 % gel is shown in Fig. 4. It can be seen that a number of protein fractions can be resolved and that the scanning procedure is applicable to the study of tissue protein separations. The presence of a large amount of material absorbing at 280 nm and migrating with the electrophoretic front precludes the application of the method to the study of those brain proteins which also migrate with the electrophoretic front, *e.g.*, the S-100 protein[11]. The nature of this front peak and its constituent(s) are not yet known. The presence of this artifact was also observed in liver homogenates, and it was verified that the origin of this peak was the tissue sample. In some preliminary experiments, dialysis or activated charcoal treatment of the sample prior to electrophoresis were unable to eliminate this peak. Protein components of brain and liver with slower electrophoretic mobilities can, however, be satisfactorily resolved and quantitated using crystalline albumin as a standard.

The use of albumin as a standard for the quantitation of serum and tissue protein fractions has certain disadvantages. Since the absorption of protein at 280 nm is mainly due to the tryptophan and tyrosine residues, individual proteins could exhibit different specific absorbancies at this wavelength, depending upon their tryptophan and tyrosine content. Any quantitative estimate of proteins based on the extinction of albumin would thus be erroneous. A similar line of reasoning is also applicable to

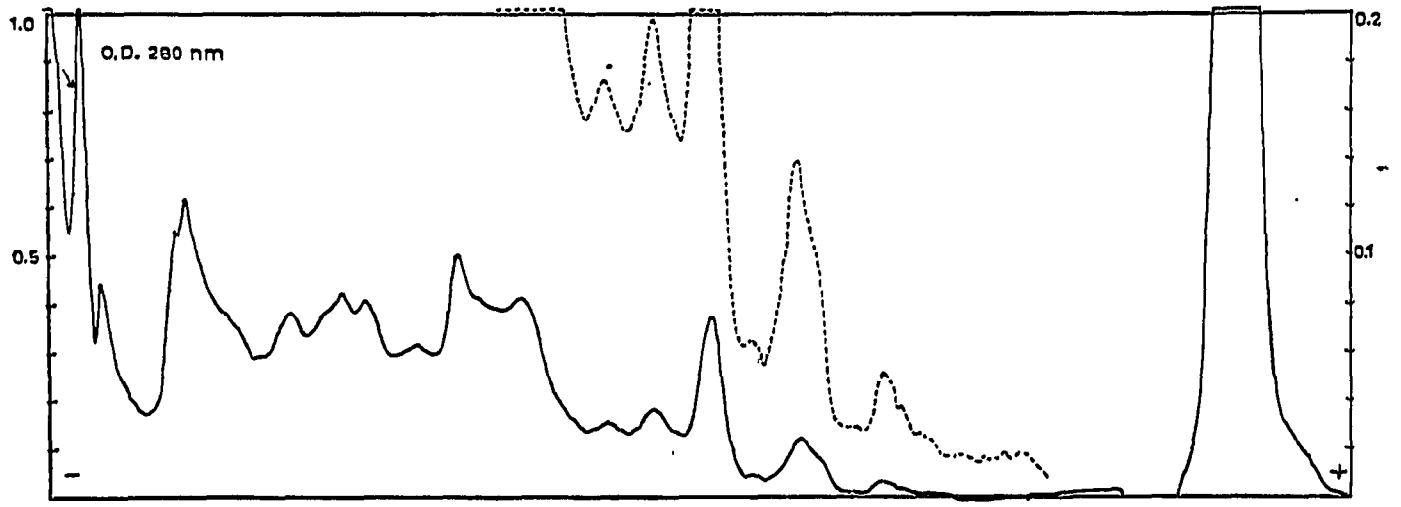

Fig. 4. Scan of the separation of rat brain stem protein in 11.2 % acrylamide gel at 280 nm. The arrow indicates the beginning of the separation gel. The continuous and broken lines represent scans at settings of 1.0 and 0.2 optical density unit, respectively. The large anodal peak corresponds to an artifact originating from the sample.

gels stained with amido black. Due to different binding capacity of the dye with different proteins, quantitation of proteins based on the staining capacity of albumin as a standard is also subject to error. An additional disadvantage of amido black staining was the lack of proportionality between the amount of protein and the intensity of the stain over the range of albumin concentrations tested (1–50 μg). However, a relative estimate of protein fractions in a sample can be obtained with albumin as the reference. It should be noted that the high sensitivity of amido black staining facilitates the measurement of amounts of protein smaller than those in the lower limits of the present procedure.

In light of the above limitations and the results presented it is apparent that scanning the gels at 280 nm is more satisfactory than scanning stained gels. An additional advantage of U.V.-scanning is that the whole procedure can be finished in a very short time. For measurement of radioactivity the gels can be stained quickly after scanning at 280 nm with bromophenol blue to visualize the bands[12], and the bands can be sectioned.

To indicate the degree of resolution that can be obtained by the present method for scanning of protein gels, photographic reproductions of the same two gels (serum and brain stem) the scans of which are presented in Figs. 3 and 4, are shown in Fig. 5. The resolution of protein bands seemed to depend on the slit width. Slits ranging between 50 to 300 μm in width were tested. The 50-μm wide slit was found to give the best resolution without loss of sensitivity. In addition the resolution of particular

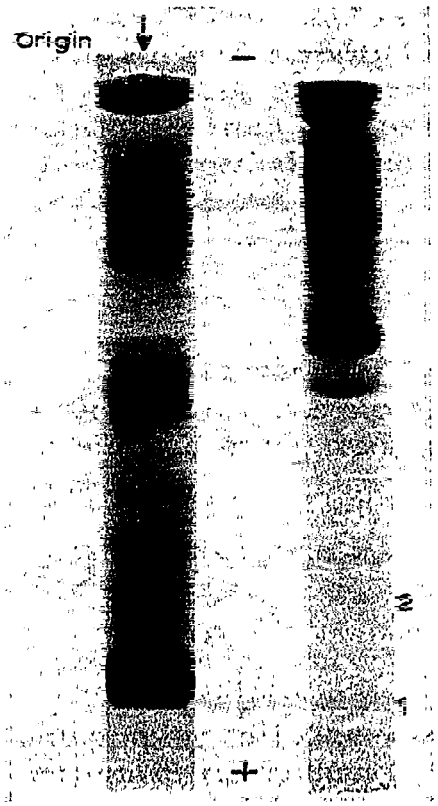

Fig. 5. Photographic reproductions of calf serum and rat brain stem protein separations. Left: Calf serum protein separation in 7.5% acrylamide gel. Right: Rat brain stem protein separation in 11.2% acrylamide gel. Bands 1 and 2 were obscured by the large anodal peak when the unstained gel was scanned at 280 nm as shown in Fig. 4.

protein bands can be improved by altering the time and/or other parameters of electrophoresis. The scanning procedure can also be adopted for any spectrophotometer in which the light source, the monochromator and the photomultiplier are arranged linearly.

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