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# QUANTITATIVE ELECTROPHORESIS IN POLYACRYLAMIDE GELS OF 2–40 %

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SUMMARY

The concentration of acrylamide, the concentration of the crosslinking agent bisacrylamide, and the polymerization conditions affect the structure of polyacrylamide gels and their suitability as molecular filters. Evidence is presented that a change in acrylamide concentration tends to affect the average "pore" size of the gel while changes in bisacrylamide concentration tends to affect the maximum "pore" size. Evidence is presented that bisacrylamide can act as a chain termination agent. Instructions are provided for preparing excellent molecular filters from 2 % acrylamide to 40 % acrylamide gels.

## INTRODUCTION

It has been pointed out by BLATTLER AND REITHEL[1], BLATTLER[2], KINGSBURY AND MASTERS[3], PARISH AND MARCHALONIS[4], HEDRICK AND SMITH[5], and by CHRAMBACH AND RODBARD[6] that protein molecular weights can be obtained by carrying out electrophoresis experiments in polyacrylamide gels with different pore sizes.

The purpose of this paper is to investigate the properties of polyacrylamide gels so that quantitative molecular weight studies can be carried out in the most suitable gel. Gels were characterized with respect to two variables: acrylamide and bisacrylamide concentration. Gels were prepared ranging from very low density, 2 % acrylamide, to very high density, gels of 40 % acrylamide. The concentration of bisacrylamide also has dramatic effect on the properties of these gels and these effects were studied over a wide range of concentrations.

The properties of the gels were characterized by utilizing protein standards of widely varying molecular weights. Gels were judged to be useful also on the basis of their gross physical characteristics. A useful gel must be sufficiently strong to be handled, stained, photographed and stored.

## EXPERIMENTAL

### *Materials and methods*

The electrophoresis experiments were carried out in a vertical gel electrophoresis unit. This unit and its operation were described by BLATTLER[7]. An improved version

of this equipment, the Model A electrophoresis unit, was obtained from the Biochemical Instrument Co.*. The gels were held between two thin glass plates. The temperature outside the glass plates was regulated to a constant 17° with a mercury contact thermometer and a relay obtained from Cole-Parmer Instrument Co. The temperature inside the gel was monitored with a Versitherm meter and a needle-type temperature probe also from Cole-Parmer. One out of the ten channels available for samples was left empty as a control and this channel was used for inserting the needle temperature probe.

In order to obtain precise results, the experimental variables in electrophoresis, namely pH, temperature and voltage, were carefully controlled. Each variable was checked at the beginning, middle, and end of each experiment. The pH of the anode and cathode was maintained at pH 7.4 ± 0.1 during the whole experiment. The applied voltage to the instrument was always 270. The voltage drop across the gel was directly measured each time. There is a voltage drop across the buffer chambers of 40 to 60 V. The voltage across the gel varies with the density and resistance of the gel. A 7% gel has a potential of 14 V per cm while a 40% gel had a potential of 16.6 V per cm.

The protein standards were obtained from Miles Laboratory. The acrylamide and bisacrylamide were from Kodak. The tetramethylethylenediamine (TMED) was from Baker. All other chemicals were reagent grade. The electrophoresis buffer contained 0.001 *M* $Na_3HEDTA$, 0.0054 *M* $Na_2HPO_4$, 0.0013 *M* $KH_2PO_4$ and 0.1 *M* glycine. The resulting pH was 7.36 to 7.40.

Gels were prepared on the basis of weight of acrylamide to volume of buffer. Thus, a 5% gel contains 5 g of acrylamide dissolved in water to 100 ml. This does not include the small amount of bisacrylamide added for crosslinking purposes. Polymerization was accomplished by dividing the solution into two beakers in portions of 100 ml and 10 ml. The 100-ml portion was heated almost to boiling and 40 µl of TMED were added. To the unheated 10-ml portion, 0.2 ml of 5.0% ammonium persulfate was added. Oxygen was removed from these two portions by bubbling nitrogen through them within a nitrogen-filled glove bag. The smaller portion containing the $(NH_4)_2S_2O_8$ was then added with further bubbling to the larger portion containing the TMED and the mixture was poured into the gel plate holder. The sample mold was inserted and polymerization was allowed to proceed for 30 min. Heating of the acrylamide solution before polymerization encouraged complete polymerization in a short time and also drove out some of the dissolved oxygen. For gels of higher concentration than 10%, the acrylamide solution was heated only to about 60° so that polymerization did not occur before the solution could be poured into the mold. For gels of higher concentration than 25%, the light from a 60-W bulb was allowed to shine on the solution in the mold instead of heating the solution. In these same gels, heat was dissipated from the glass walls of the mold during polymerization by a stream of nitrogen to prevent cracking the glass. Gels higher than 40% are difficult to make due to heat production. In a second series of experiments, the oxygen was removed by bubbling nitrogen through a gas washing bottle equipped with a fritted disc at the bottom. It is necessary to degas the nitrogen from the solution by applying a vacuum. In the more dense gels, sufficient heat is produced to

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drive the excess nitrogen from solution and to form bubbles in the gel. Degasing avoids this difficulty.

After polymerization, the sample mold was removed from the gel. Before adding the sample, 250 V were applied to the gel for at least 5 min to remove excess ammonium persulfate from the sample application area. Samples were not added until thermal equilibration was reached.

## RESULTS

Fig. 1, curve A, is a plot of the bisacrylamide concentration *vs.* acrylamide concentration for Cyanogum 41. This commercial preparation is widely used and contains 5 % by weight of bisacrylamide. The two arrows indicate the limits for obtaining usable gels with this material.

Fig. 1, curve B, is a similar plot where the bisacrylamide concentration is held

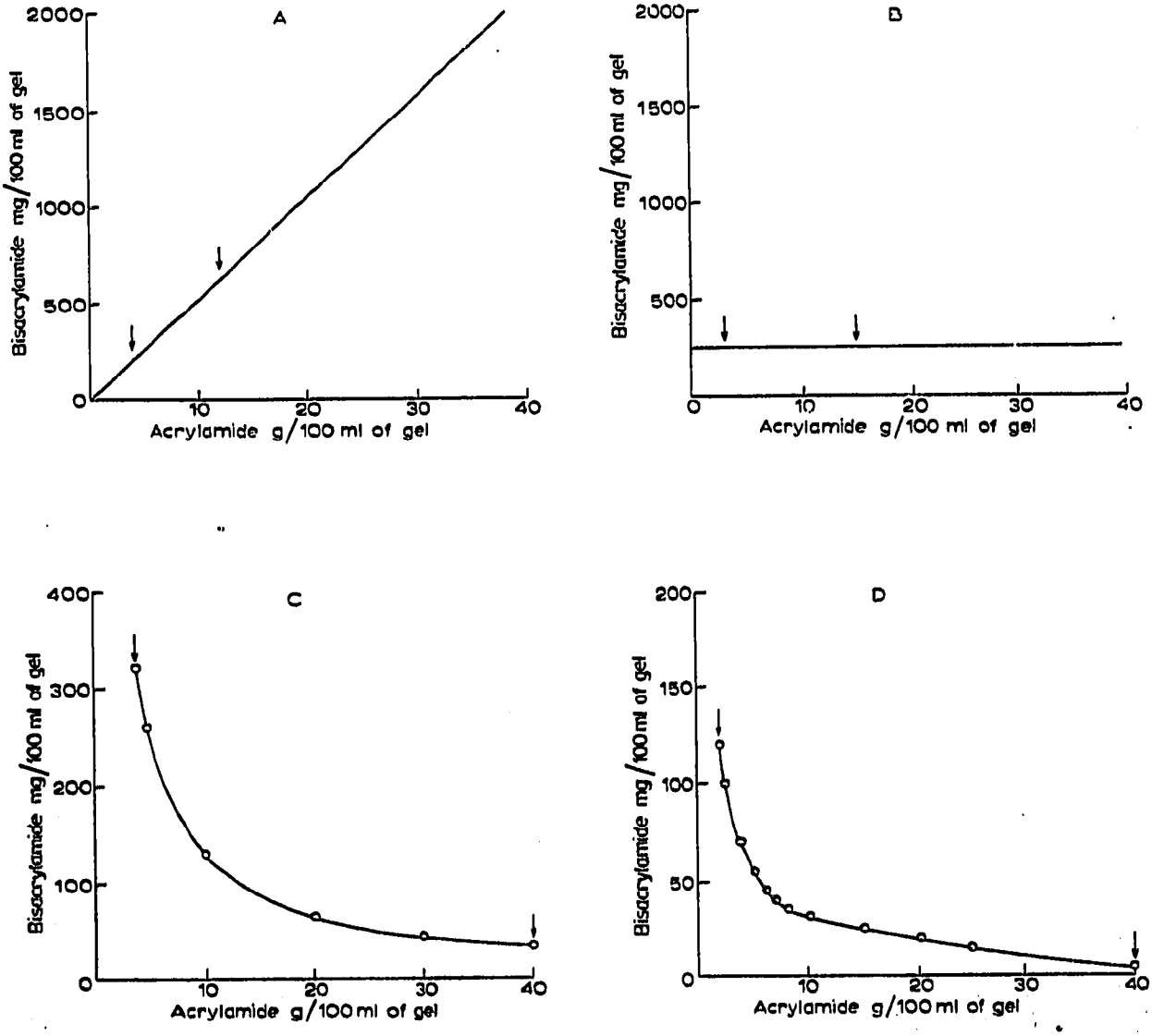


Fig. 1. Plots of bisacrylamide concentration *vs.* acrylamide concentration in gels. The four graphs show different ratios of bisacrylamide to acrylamide that produce gels suitable for electrophoresis. The interval between the two arrows indicates the range over which the ratio produces good gels.

constant over the whole range of acrylamide concentrations. The two arrows indicate the practical limits between which usable gels are obtained.

Fig. 1, curve C, is a similar plot where the ratio of bisacrylamide to acrylamide is described by the following formula:

$$(\text{Bisacrylamide, mg}) = \frac{\text{Constant}}{(\text{Acrylamide, g})} \qquad (1)$$

The constant has a value for curve C of 1300 mg bisacrylamide·g acrylamide. The arrows at 4 % and 40 % indicate that the formula provides stable gels with good handling properties over this wide range.

In Fig. 1, curve D, is an empirical curve which yields good gels over a range from 2 % to 40 %. The gels at 2 % are slippery and watery, but remarkably stable considering the low gel concentration. The gels at 40 % are supple and strong.

In Fig. 2, the cross-hatched area indicates the area between curve C and curve D. Any ratio of bisacrylamide to acrylamide found in this area will produce a good gel with desirable handling properties.

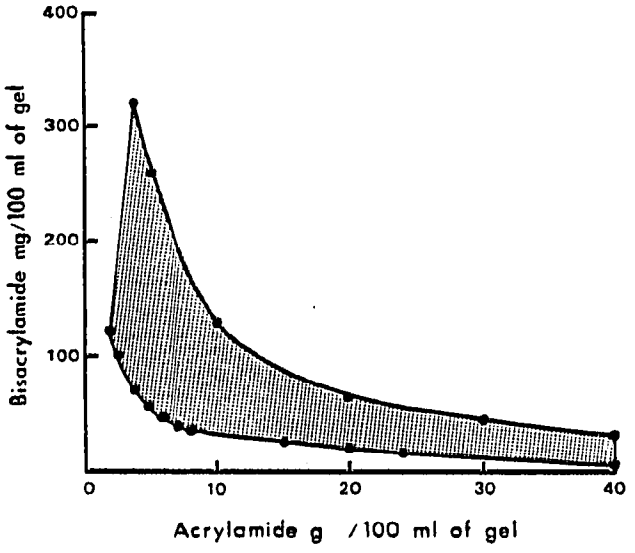


Fig. 2. Graphs C and D of Fig. 1 are superimposed. Any point between the two graphs will produce a gel with suitable handling properties for electrophoresis.

The physical properties of these gels are of interest. A 15 % gel formulated according to curve A is so brittle as to be almost unusable. A 15 % gel on curve C or D is supple and strong. A gel containing 2 g of acrylamide and 120 mg of bisacrylamide is extremely slippery and flexible, but relatively strong. A gel containing 2 g of acrylamide and 645 mg of bisacrylamide is milky, opaque-white and very paste-like.

Fig. 3 shows six gels which differ from one another only by the concentration of acrylamide and bisacrylamide. The ratio of bisacrylamide is as described by formula (1) or curve C of Fig. 1. Gel A is a 5 % gel; B is 10 %; C is 20 %; D is 30 %; E is 35 %; and F is 40 %.

Fig. 4 shows how the electrophoretic mobilities of various proteins respond to changes in gel density as described by formula (1). The top curve shows the results

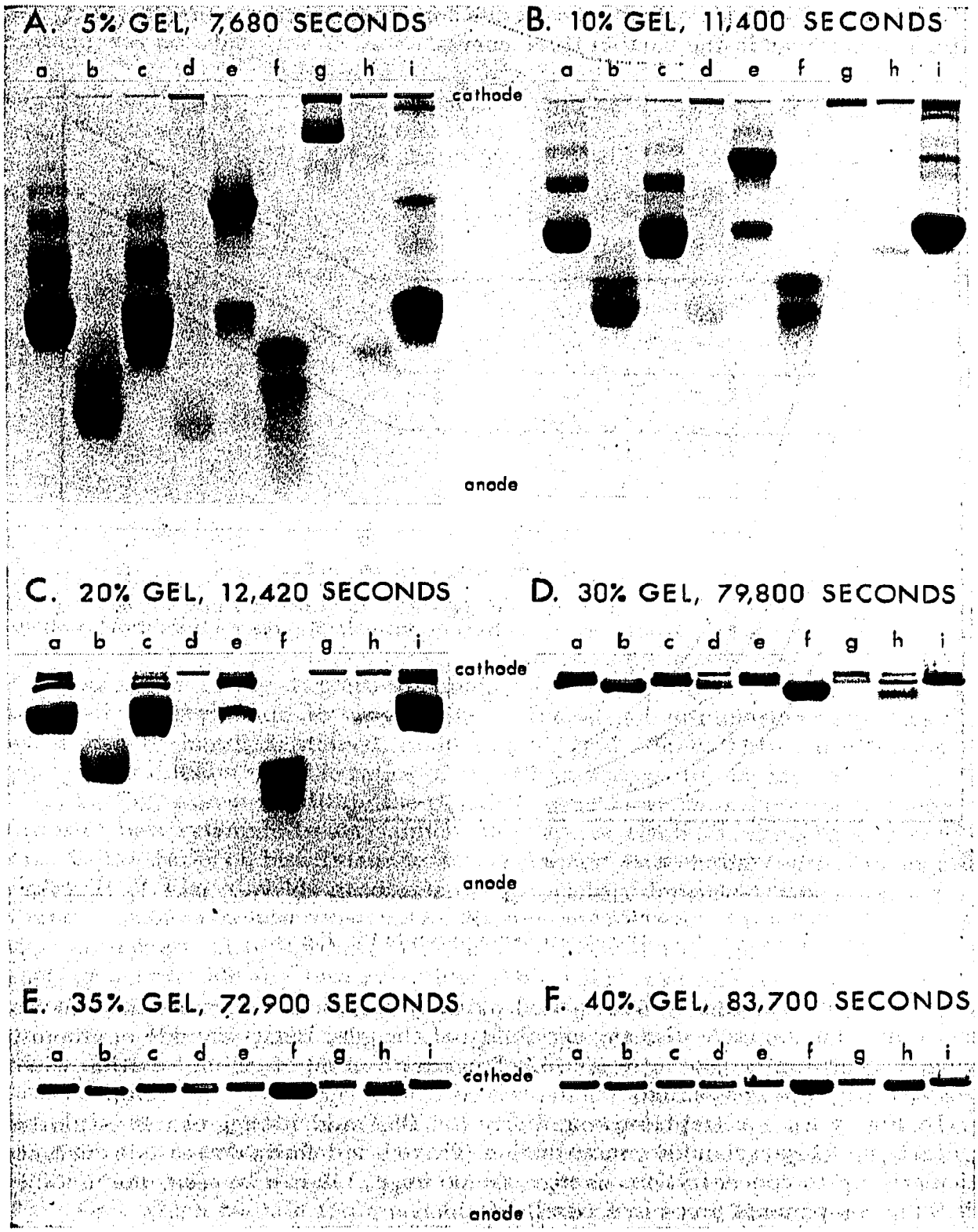


Fig. 3. Electrophoresis of six gels differing from one another only by the concentration of acrylamide and bisacrylamide. Column $a$ = BSA; $b$ = ovalbumin; $c$ = human serum albumin; $d$ = human glycoprotein; $e$ = human transferrin; $f$ = lactoglobulin; $g$ = porcine thyroglobulin; $h$ = lactalbumin; $i$ = normal human serum. Column $j$ is not shown since it was left blank as a control and was also used for a site to measure the internal gel temperature.

for ovalbumin. Fig. 4 also shows the results for bovine serum albumin (BSA) and its dimer and trimer in the bottom three curves.

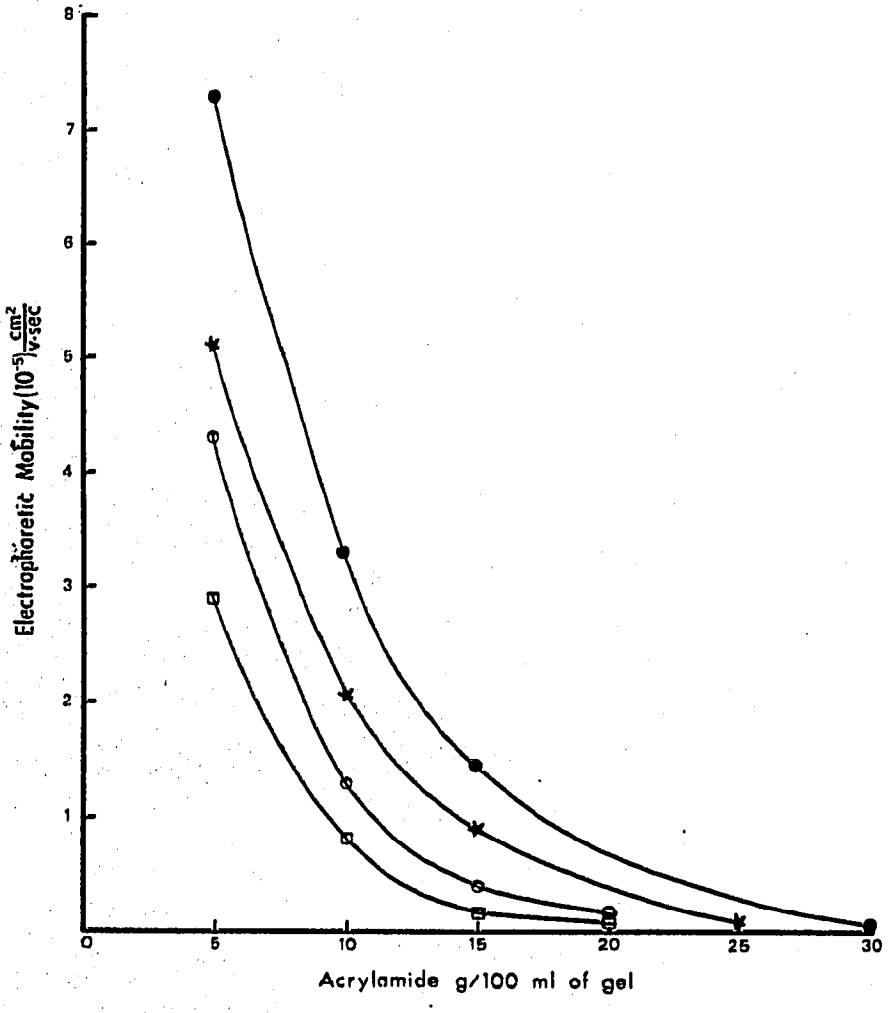


Fig. 4. Electrophoretic mobility of ovalbumin (●), BSA monomer (★), BSA dimer (○), and BSA trimer (□), as a function of acrylamide concentration. Gels were formulated according to curve C of Fig. 1. The data are taken from the experiments presented in Fig. 3.

A series of experiments was carried out in 7% gels to determine the effect of bisacrylamide on the molecular sieving ability of the gels. Bisacrylamide in amounts varying from 10 mg to 500 mg was utilized in each 100 ml of gelling solution which also contained 7 g of acrylamide.

In Fig. 5 the electrophoretic mobility for BSA and its aggregates is plotted as a function of bisacrylamide concentration. There is a definite decrease in mobility particularly up to concentrations as high as 200 mg %. As can be seen, the mobility of all three components drops in a parallel fashion.

## DISCUSSION

The experiments described here were designed to elucidate some of the basic parameters of gel structure important to quantitative polyacrylamide electrophoresis.

The experiments were designed so that precise control of temperature, pH and voltage was possible.

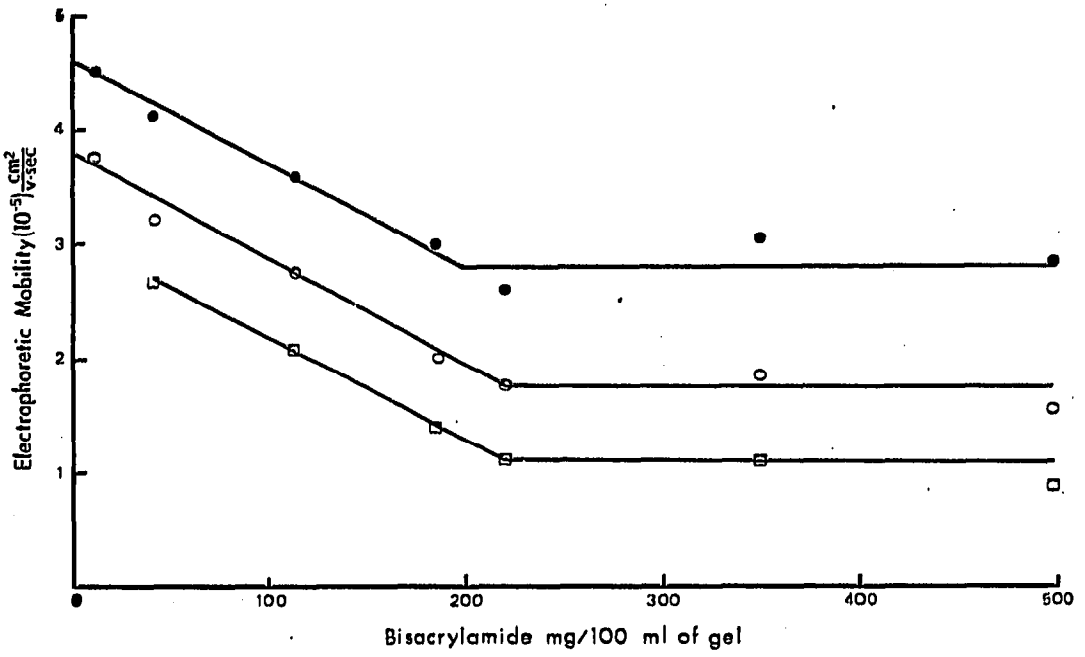


Fig. 5. Electrophoretic mobility of BSA (●), BSA dimer (○), and BSA trimer (□) in 7% acrylamide gels where the bisacrylamide concentration varies from 10 mg% to 500 mg%.

As shown in Fig. 1, polyacrylamide gels can be formulated with widely varying ratios of bisacrylamide to acrylamide. Practical gel formulations must yield gels which can be handled, stained, photographed and stored. This consideration alone puts constraints on possible ratios of crosslinking agent to polymer residue.

In Fig. 1 arrows indicate the practical outer limits for minimum and maximum ratios of bisacrylamide to acrylamide. In graph A the limits are 4 % and 12 % gels. This is the ratio of bisacrylamide to acrylamide one obtains with Cyanogum 41. Graph B of Fig. 1 yields good gels over a slightly improved range, 3 % to 15 %. Graph C yields gels with suitable handling properties from 4 to 40 %. Curves of this type are generated by formula (1). Our selections for the constant in formula (1) is 1300 (g acrylamide·mg bisacrylamide). This value was selected because good gels are obtainable over a wide range and these gels have desirable molecular sieve properties. However, for gels of 2 to 4 %, the ratio of bisacrylamide is too high when obtained from graph C. In general, low density gels require higher ratios of bisacrylamide; however, too much bisacrylamide produces weak, paste-like gels. Graph D was empirically obtained and yields good gels from 2 to 40 %. The handling properties are very good, but molecular sieving properties are generally less satisfactory than those of graph C over the range from 4 to 40 %.

Gels which contain too much bisacrylamide have a milky-opaque color. Thus, a milky gel is a good indication that excessive amounts of bisacrylamide are present. A 5 % gel containing 250 mg % of bisacrylamide shows a faint tinge of milky color. A 2.5 % gel containing 645 mg of bisacrylamide is milky in color and very paste-like. Gels which contain an excessive amount of bisacrylamide are rigid, but also brittle. For example, 15 % gels formed from Cyanogum contain 750 mg of bisacrylamide and

14.25 g of acrylamide in 100 ml. These gels are extremely brittle and completely impractical for normal use.

The significant point is not that these gels are rigid, but that they are brittle. If they were formed from any long-random polymers, one would not expect this brittleness. These gels are probably formed from many short chains with some of these chains interconnected by crosslinks formed from bisacrylamide.

It is clear that the role of bisacrylamide in these gels is more than serving as a crosslinking agent or as a monomer unit in the polymer. It is likely that bisacrylamide has a relatively high tendency to serve as a chain terminator. By thus producing short chains, one would obtain paste-like gels in low-density gels or brittle gels in high-density gels. Bisacrylamide could serve as a chain terminator through two possible mechanisms. The bulky side group could provide steric hindrance, thus slowing down the rate of access of suitable vinyl reactants while not hindering small free radical scavangers. The second possibility is that the free radical on the end of the chain could react with the other vinyl tail of the bisacrylamide causing cyclization, followed by chain termination by a free radical scavanger.

The two effects of bisacrylamide are opposite in nature. When bisacrylamide crosslinks two adjacent polymers, it stabilizes the material into a definite gel structure. As more bisacrylamide is added, it becomes increasingly likely that if two chains are adjacent, they are crosslinked. Thus, bisacrylamide addition produces increasingly rigid structures with decreasing "pore" sizes; however, as more and more bisacrylamide is added, less useful results will be obtained per unit of bisacrylamide added, since the early additions have saturated the significantly useful sites. Thus, the benefits of adding extra bisacrylamide exist only up to a certain point. This can be seen experimentally in Fig. 5. As the concentration of bisacrylamide in 7% gels increases from 10 mg% to 200 mg%, there is a marked reduction in the mobility of the proteins. However, additional amounts of bisacrylamide up to 500 mg% produce no additional reduction in mobility. Even more significant, if bisacrylamide causes chain termination and short polymer chains, then as one adds extra bisacrylamide, structural weakness should be introduced. Thus, these two effects of bisacrylamide are opposing.

Fig. 2 shows the practical range of values for the ratio of bisacrylamide to acrylamide. Higher values of bisacrylamide will, in some cases, yield adequate gels; however, we feel that curve C of Fig. 1, which is the top curve of Fig. 2, comes closest to prescribing the optimum gel for most purposes over the widest range of gels that any simple formula can provide.

We have preliminary evidence that catalyst conditions are a particularly important factor in producing uniform high-quality gels. KINGSBURY AND MASTERS have shown that carbonic anhydrase isoenzymes have higher mobility rates in gels formed with high catalyst concentrations. For these reasons, we carefully specified catalyst conditions in MATERIALS AND METHODS. In comparison studies where the gel concentration is varied, it is hard to standardize gelling conditions. This is because heat accelerates the reaction and the reaction itself generates considerable heat. In spite of considerable attention paid to polymerization conditions, this factor is still subject to the most variation in our experimental system.

An increase in the concentration of acrylamide produces effects which can be distinguished from an increase in the concentration of bisacrylamide. In general,

an increase in either component reduces the mobility of the various proteins both large and small. Fig. 4 shows the effect for acrylamide. The ratio of bisacrylamide to acrylamide is described by curve C. These curves show the strong influence of increasing acrylamide concentration on proteins which are small compared to the maximum "pore" size.

Bisacrylamide produces a striking effect on the maximum "pore" size of the gel. For example, in 20 % gels an increase of bisacrylamide from 20 mg % to 65 mg % can reduce the maximum "pore" size from 350,000 to 210,000 where the "pore" size is represented by protein molecular weights. The effect of bisacrylamide on proteins mobilities whose size is much smaller than the maximum "pore" size is much less dramatic. For example, in Fig. 5, where results for BSA monomer are presented, a twenty-fold increase in bisacrylamide concentration produces only a 33 % reduction in mobility.

## CONCLUSION

The average "pore" size of acrylamide gels is strongly influenced by the acrylamide concentration. The maximum "pore" size is strongly influenced by the bisacrylamide concentration. For a given gel concentration there is an optimal ratio of bisacrylamide to acrylamide. Good polyacrylamide gels can be produced by curve C of Fig. 2 from 4 to 40 % and curve D of Fig. 2 enables one to produce gels down to 2 %. Other researchers have demonstrated the importance of polymerization conditions on gel structure. Comparison of many experiments indicate that gels containing the optimum amount of bisacrylamide produce superior resolution and band sharpness for even the smaller proteins. This is true even though the most dramatic effects of bisacrylamide are on the larger proteins, whose size approaches the maximum "pore" size of the gel. By controlling the amount of the monomer and crosslinking agent, the average "pore" size and maximum "pore" size of the gel can be influenced. Thus, an optimum gel can be created for each purpose.

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