ZONE ELECTROPHORESIS IN COLUMNS OF AGAROSE SUSPENSIONS

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INTRODUCTION

Agar gel electrophoresis is a frequently used analytical tool, but is rarely used for preparative purposes, owing partly to the difficulty of localizing the zones in the gel without utilizing a denaturing staining technique, and partly to the rather cumbersome and tedious work required to elute the zones from the gel by repeated freezing and thawing. Both these disadvantages can be almost entirely eliminated, however, if a *suspension* of gel particles is utilized as a supporting medium instead of a coherent gel mass. As this electrophoresis method has been but briefly described before[1,2], it will now be treated in more detail. The experiments were originally made in suspensions of agar, but, owing to the many acidic groups in this polysaccharide, a series of undesirable phenomena arose. During a search for other more neutral anticonvection agents, it was found that agarose gave more satisfactory results. An important characteristic of agarose suspensions is that they show extremely low adsorption of the substances to be separated[1].

Owing to the characteristic flow properties of gel suspensions, the electrophoresis columns can be emptied with negligible zone spreading; this is a very important advantage of these systems.

MATERIAL AND METHODS

Preparation of agarose

For the preparation of agarose, a method based on that of ARAKI was originally used[1,3]. In addition, a simpler method, more suitable for larger scale preparations has been developed[4], based on the fact that acidic polysaccharides precipitate in the presence of quaternary ammonium compounds, leaving the neutral agarose in solution. This procedure also has the advantage that the product obtained has a higher gelling ability and can therefore be used in lower concentrations for the preparation of the gel suspension (see the next section). The agarose used in the experiments represented in Figs. 7 and 8 was prepared according to the new method; the other experiments were performed before this method was introduced.

Preparation of suspensions of agarose

The agarose is dissolved by boiling in a buffer volume somewhat larger than the volume of the electrophoresis tube (the same buffer is used as in the electrode vessels). The solution is allowed to cool without stirring in a stoppered Erlenmeyer flask at room temperature. The next day the gel is broken into small pieces by swirling the flask or

by gentle stirring; this agitation of the gel should proceed only for 1–2 min. If a film has formed on the surface of the gel, this film should first be removed. The agarose suspension should be de-aerated for some minutes before the electrophoresis tube is filled.

If the agarose is prepared according to ref. 4, the suspension will for most experiments have satisfactory anticonvection properties at concentrations as low as 0.16 % (0.16 g agarose per 100 ml buffer); if the original method[1] for preparation of agarose is followed a somewhat higher concentration is in general required (0.18 %). For determination of the minimum concentration of agarose required to stabilize the sample against convection, the following procedure can be used. A series of suspensions of different agarose concentrations (*e.g.* 0.13, 0.15, 0.17 and 0.19 %) is prepared and transferred to small test tubes. One drop of the sample solution (if uncolored it should be stained with a dye) is carefully added to each test tube. The lowest agarose concentration that prevents the sample from falling towards the bottom of the test tube, usually gives sufficient stabilization for electrophoresis, although a 0.01 % higher concentration is recommended.

Analytical procedures

The presence of agarose in the fractions obtained from the electrophoresis experiments is, in many analyses, not disturbing. Measurement of enzyme activities can thus often be carried out without removal of the gel particles[2]. Further, protein determinations by the FOLIN–LOWRY method[5] for preparation of electropherograms can be performed on aliquots of the collected, agarose-containing fractions. However, determinations of the material distribution by measurements of the ultraviolet absorption of the fractions give unreliable values owing to light scattering by the gel particles.

Removal of agarose

When it is essential to remove the agarose from the collected electrophoresis fractions, this can easily be done by centrifuging. For this purpose, simple table centrifuges can be used although larger centrifuges capable of giving 20,000 *g* are preferable. The carbohydrate concentration of the supernatant has been determined by the anthrone method[6] and was found to be 0.007 %, independent of the pH of the buffer from which the agarose suspension was prepared (only the pH interval within which most electrophoresis experiments are performed has been examined, *i.e.* 4.0–9.7). The degradation of agarose by hydrolysis is thus negligible, at least in the tested pH range.

If the gel pellet obtained after centrifuging is suspended in buffer and re-centrifuged and this washing procedure is repeated an almost 100 % yield is obtained.

ELECTROPHORESIS APPARATUS AND EXPERIMENTAL PROCEDURE

If the electrophoresis tube of an apparatus of conventional type[7] is filled with suspensions of commercial agar the suspension will move during electrophoresis towards the anode. If the anode is placed at the lower part of the electrophoresis tube, and this is closed with a dialysis membrane to prevent the easily movable suspension from running out of the tube, the suspension will be pressed towards the membrane, and the length of the agar column can be reduced as much as 50 %. The formation of cracks in the agar suspension has also been observed in such experiments. All these disturbing

effects are probably due to electroendosmosis, caused by the presence of charges in the agar, on the glass walls of the electrophoresis tube, and in the membrane.

In view of these difficulties, we studied the use of agarose instead of agar. It turned out that all the above disadvantages were considerably reduced when the suspension was prepared from this more neutral polysaccharide and the membrane was replaced by a plug of an agarose gel. A completely immobile agarose suspension was not obtained, however, until the electrophoresis tube was fitted with a "shunt" (S in Fig. 1). If this "shunt" is filled with buffer and the electrophoresis tube with agarose suspension, the hydrodynamic resistance in this "shunt" is lower than in the electrophoresis tube. The electroendosmotic reflow will consequently pass through the "shunt" and not through the electrophoresis tube. The suspension will therefore not be compressed. The existence of a hydrodynamic flow in the "shunt" can easily be demonstrated by adding a neutral dye, for instance DNP-ethanolamine[8], to the buffer in the "shunt". The direction of this flow can be reversed by reversing the direction of the current.

The method to be selected for removing the agarose bed from the electrophoresis tube depends upon the diameter of the column. As a result, the apparatus having a narrow electrophoresis tube has a few structural features, which are different from the larger equipment. Construction details (in some respects similar to those introduced by PORATH[7]) are given in the following sections. All operations which do not depend on the size of the electrophoresis tube are described only in section (a).

(*a*) *Narrow columns (about 0.5 cm diam.)*

A thin membrane D (Fig. 1), moistened with buffer, is fixed to the lower part of the electrophoresis apparatus by means of tightly stretched rubber bands R. As an alternative to the dialysis membrane, a plug N of a gel of agarose or polyacrylamide can be used. The agarose plug is prepared by sucking up a warm 2 % agarose solution through the porous polyethylene discs V 1 and V 2 and allowing it to cool. These discs will be firmly fixed to the glass tube if they are stamped to a diameter somewhat larger than the inner diameter of the tube. If a plug of polyacrylamide is used, this should have the composition T = 5%, C = 3 % (for the definition of these parameters and for the preparation of such a gel, see ref. 16).

The bottom section of the electrophoresis apparatus is filled up to the "shunt" S with agarose suspension and the electrophoresis tube E and the "shunt" S are filled with buffer. The screw clip B is closed and the buffer in the electrophoresis tube is sucked away and is replaced by agarose suspension*. The sample is applied and the rest of the electrophoresis tube is filled with agarose suspension as described below under "Application of the sample" (for these narrow columns it is, however, more convenient to replace the glass tube G in Fig. 4 with a piece of polyethylene tubing of 1 mm I.D.). The side arm A is attached and buffer from the electrode vessel is sucked up through the tubing T 1. It is essential that there are no air bubbles in the tubings T 2 and T 3. This can be controlled by switching on the current and observing whether or not it increases when the screw clip B is opened.

When the electrophoresis is finished and the column is to be emptied, the screw clip B is closed, the stopcock F is opened and the side arm A is detached. Starting from

* The use of a piece of polyethylene tubing for filling the electrophoresis tube will prevent air bubbles from forming in the agarose column.

the top of the electrophoresis tube, which is graduated in cm, 1-cm fractions are sucked out with the aid of a piece of polyethylene tubing (I.D. 1 mm). It is important that the free end of the tubing is kept *just* under the surface of the agarose suspension during this operation. Each 1-cm fraction is transferred to a test tube and aliquots are used for analyses.

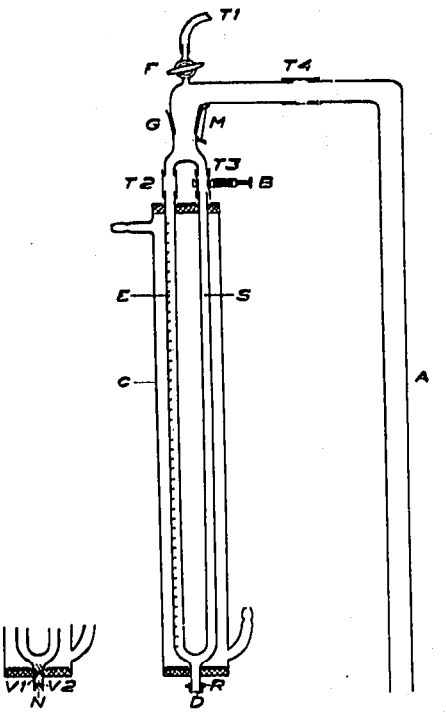


Fig. 1. Electrophoresis apparatus for narrow columns (diam. about 0.5 cm):

E = electrophoresis tube, graduated in cm
S = "shunt"
C = cooling mantle
A = side arm
D = dialysis membrane
R = rubber bands
B = screw clip
F = stopcock
G = ground glass joint
M = rubber bands
T1–T4 = polyvinyl chloride tubings

The dialysis membrane D can alternatively be replaced by a plug N of a gel of agarose or polyacrylamide fixed to the glass tube by means of the porous polyethylene[9] ("Vyon") discs V1 and V2, as shown in the drawing on the left.

(*b*) *Intermediate columns (about 0.9 cm diam.)*

These columns can be emptied in the same way as described above for narrow columns. However, the risk of remixing two adjacent zones during the sucking out procedure increases with the diameter of the column. It is therefore preferable to force the suspension out of the electrophoresis tube by pumping buffer into the top of it.

After the electrophoresis is finished, the screw clip B 1 (Fig. 2) is closed. A peristaltic pump is connected with the closed stopcock F. The screw clip B 2 is closed. The polyvinyl chloride tubing T 5 is cut up and the Y-tube Y is turned aside. During this procedure the tubing T 5 must be surrounded by the buffer in the electrode vessel, otherwise there is a risk that air bubbles will enter the electrophoresis tube and blurr the zones. For the same reason it is important that the apparatus is mounted so that the electrophoresis tube is not inclined. The electrode vessel is removed after its buffer

has been sucked away, the stopcock F is opened and the peristaltic pump is started. Flow rates of about 10 ml/h have been used for these intermediate columns.

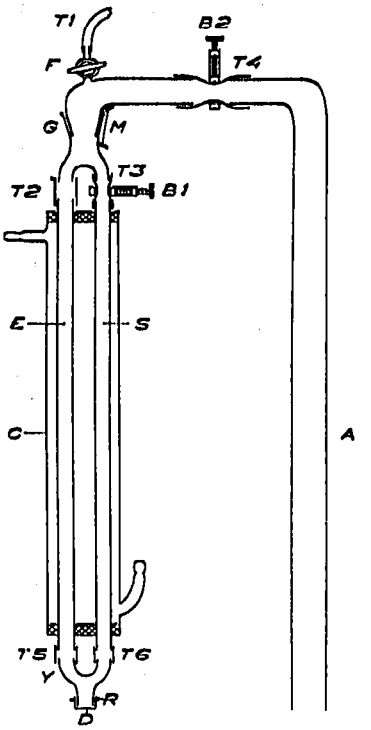


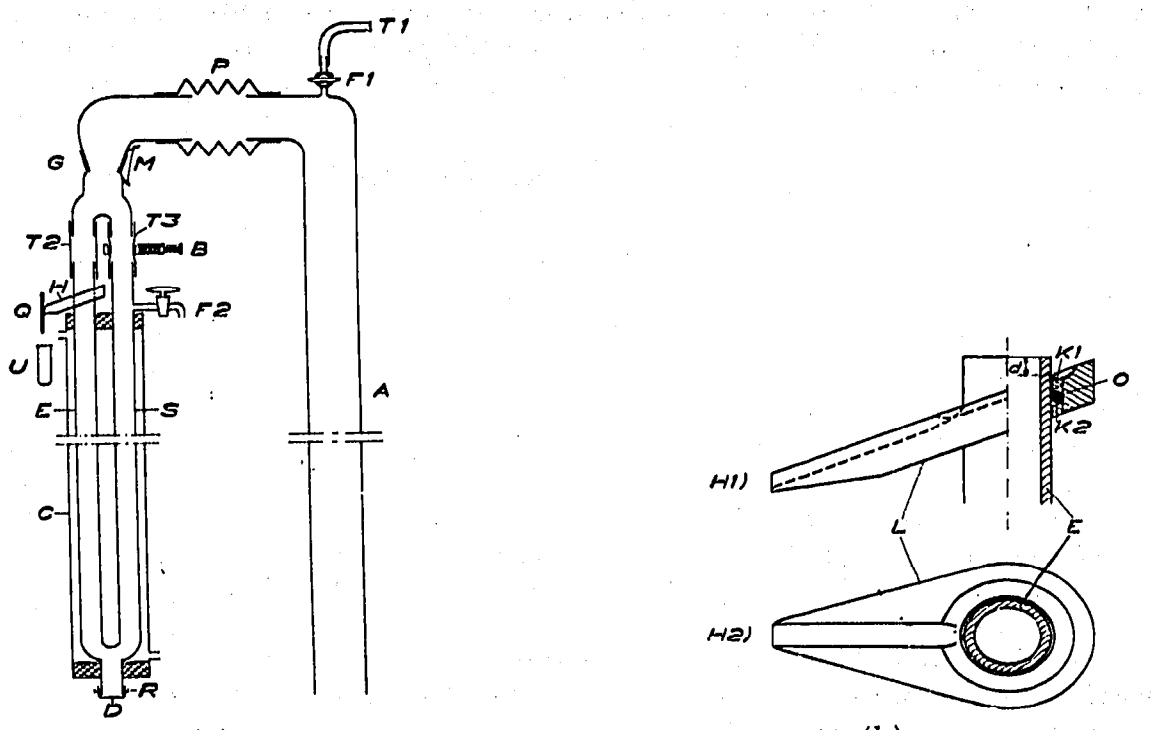
Fig. 2. Electrophoresis apparatus for intermediate columns (diam. about 0.9 cm):

E = electrophoresis tube
S = "shunt"
C = cooling mantle
A = side arm
D = dialysis membrane
R = rubber bands
B1, B2 = screw clips
F = stopcock
G = ground glass joint
M = rubber bands
Y = Y-tube
T1–T6 = polyvinyl chloride tubings

(c) *Wide columns (about 2 cm diam.)*

As described above, intermediate columns are emptied by pressing the agarose suspension downwards through the electrophoresis tube. A similar technique can be used for wider columns only if the outlet of the electrophoresis tube is modified, for instance by being supplied with a perforated disc (without this modification the suspension will run out of the electrophoresis tube as soon as the membrane has been removed and the outlet is no longer surrounded by buffer). When the zones pass the modified outlet, however, they will be distorted and so another method for emptying wide columns has been utilized:

When the electrophoresis is finished, the screw clip B (Fig. 3a) is closed. The stopcock F 1 is opened and the polyvinyl chloride tubing T2 is cut up and removed. The drainage device H is placed so that the distance d in Fig. 3 b is approximately 1 cm. The peristaltic pump is connected to the closed stopcock F 2. This is opened at the same time as the pump is started. The agarose suspension is forced up through the electrophoresis tube and runs down its walls into the drainage device H and drops into the test tubes U of a fraction collector via a glass rod Q.



(a) (b)

Fig. 3a. Electrophoresis apparatus for wide columns (diam. about 2 cm):

E = electrophoresis tube
S = "shunt"
C = cooling mantle
A = side arm
D = dialysis membrane
R = rubber bands
B = screw clip
F1, F2 = stopcocks
G = ground glass joint
M = rubber bands
T1–T3 = polyvinyl chloride tubings
P = corrugated perbunan tube[7]
H = drainage device (for details see Fig. 3b)
Q = glass rod
U = test tube in a fraction collector

Fig. 3b. Drainage device: H 1) = side view; the O-ring, O, lies in a track, formed by two obliquely cut perspex tubes K1 and K2, glued to the perspex plate L. The position of the drainage device on the electrophoresis tube E during the emptying procedure should be such that the distance d is about 1 cm. H 2) = top view.

(*d*) *Application of the sample*

About 3/4 of the water-cooled* section of the electrophoresis tube is filled with agarose suspension, as described above. The sample, previously dialyzed against the buffer, is diluted (1:1) with agarose suspension and sucked up into a syringe S via a glass tube G (Fig. 4). By turning the screw C the sample is carefully pressed out of the glass tube, which during the whole application procedure is slowly raised so that the bent orifice lies just in the surface of the suspension. It is important that the sample is evenly distributed across the agarose suspension, so that a sharp boundary is formed between the starting zone and the gel bed. The sample is covered by a 3-cm layer of agarose suspension in a manner similar to that used for the application of the sample. The remainder of the electrophoresis tube can then be more rapidly filled with suspension from a pipette.

* The temperature of the cooling water has in all experiments been 8–9°, except in that corresponding to Fig. 10, where it was 0.5°.

If the addition of agarose suspension involves an undesirable decrease in concentration of the sample, the application can be performed by injection instead of layering. In this case the glass tube G is lowered into the agarose column and the dia-

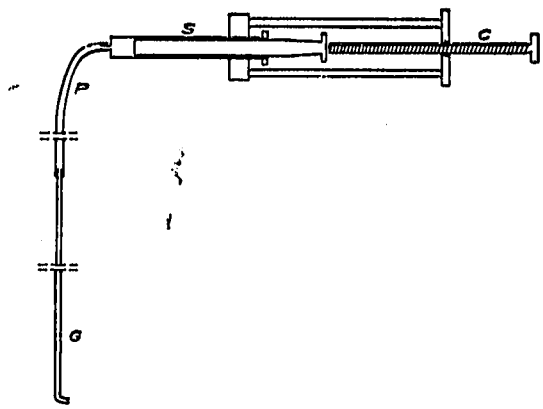


Fig. 4. Arrangement for application of the sample: S = syringe; P = polyethylene tubing; G = glass tube bent orifice; C = screw.

lysed sample (not mixed with agarose) is carefully pressed out and evenly distributed. Application of the sample by layering, is, however, preferable to that by injection.

As an exercise in application of the sample, the use of dyes, for instance pH-indicators, is recommended.

EXPERIMENTS AND RESULTS

Comparison between migration velocities in agarose suspension and in free solution

The migration velocities in 0.025 *M* sodium phosphate buffer, pH 7.3, of some proteins of different molecular size were determined in a free zone electrophoresis apparatus[10], where the diameter of the revolving uncooled electrophoresis tube was about 4 mm. These experiments were repeated in the same apparatus, with the only difference that the electrophoresis now was conducted in a 0.15 % agarose suspension, prepared in the same buffer. The current was the same in all experiments and equal to 2.5 mA. As the conductivities $\varkappa$ of the buffer alone and of the agarose suspension are different, the

TABLE I

COMPARISON BETWEEN MIGRATION VELOCITIES IN FREE SOLUTION AND IN AGAROSE SUSPENSION. Buffer: 0.025 *M* sodium phosphate, pH 7.3. The concentration of the agarose suspension prepared in the same buffer, was 0.15 %. The conductivity ratio of buffer solution to agarose suspension ($\varkappa_1/\varkappa_2$) was 1.05.

Protein	V_1 = *measured migration velocity in buffer solution* (*cm/sec* · 10^{-4})	V_2 = *measured migration velocity in agarose suspension* (*cm/sec* · 10^{-4})	$V_1\varkappa_1/V_2\varkappa_2$
R-phycoerythrin (mol.wt. 290,000)[11]	8.12	8.30	1.03
R-phycocyanin (mol.wt. 135,000)[11]	4.18	4.33	1.02
Human serum albumin (mol.wt. 68,000), stained with bromphenol blue	7.16	6.67	1.13
Cytochrome *c* from horse heart (mol.wt. 13,000)	3.58	3.76	1.00

measured migration velocities V_1 and V_2 (see Table I) should be transformed to those in the unit field to be comparable. At constant current the latter velocities are proportional to $V \cdot \varkappa$. The ratio between these in free solution and in agarose suspension ($V_1\varkappa_1/V_2\varkappa_2$) is given in the table.

Zone broadening during emptying of the column

This experiment was carried out with an electrophoresis apparatus of an older type with a detachable, not water-cooled "shunt". The dimensions of the electrophoresis tube were 0.9 × 50 cm*. The supporting medium was a 0.16 % agarose suspension, prepared in sodium acetate buffer (pH 4.85, ionic strength 0.01), to which calcium chloride was added to a concentration of 0.005 *M*. Two colored zones were applied, one containing a protein, R-phycoerythrin[11], and the other a cell suspension of *Chlorella pyrenoidosa*. The column was emptied by pumping buffer into the top of the electrophoresis tube, as described previously for intermediate columns. The flow rate was adjusted to 10 ml/h**. From the photographs in Fig. 5, taken at different intervals during the emptying, it is evident that this procedure does not cause a detectable broadening of the zones. In this experiment, the zone or *Chlorella* was introduced in order to have a zone in which the broadening due to diffusion would be negiglible.

Electrophoresis of low molecular weight substances

The same electrophoresis apparatus was used as in the previous experiment. The composition of the supporting medium was also the same. About 1 mg each of DNP-aspartic acid, DNP-serine and ε-DNP-lysine was added to 2 ml agarose suspension, which was then heated to boiling. After cooling, 0.4 ml of this sample was applied. A zone of the neutral marker substance DNP-ethanolamine[8] was introduced some centimeters above the sample zone. The run was performed at 6 mA. After 3 hours' electrophoresis the DNP-amino acids were well separated and the emptying procedure started (details are as described above for intermediate columns). Fractions of 0.5 ml were collected and were diluted to 1.0 ml before being submitted to spectrophotometric analysis at 360 mμ in a 1-cm cuvette. The electropherogram is seen in Fig. 6a. The photograph in Fig. 6b was taken just before the column was emptied.

Electrophoresis of proteins

(*a*) *Fractionation of serum on a 0.5-cm column.* The electrophoresis tube (0.5 × 55 cm, Fig. 1) was filled with a 0.17 % agarose suspension, prepared in sodium veronal buffer, pH 8.6, ionic strength 0.05. About 75 μl dialyzed normal human serum was mixed with an equal volume of the agarose suspension and applied at a distance of 10 cm from the top of the cooling mantle. After 17 hours' electrophoresis at a current of 5.5 mA the column was emptied, as described above for narrow columns, by sucking out 1-cm fractions, (*i.e.* about 180 μl). Aliquots of 100 μl were used for protein determination according to the method of FOLIN AND LOWRY[5]. The electropherogram is given in Fig. 7.

(*b*) *Fractionation of serum on a 2.0-cm column.* The above experiment was repeated with an apparatus, in which the electrophoresis tube had the dimensions

* By the length of an electrophoresis tube we mean throughout this paper the length of the water-cooled section.

** Higher pump speeds can be tolerated, however. The maximal emptying rate that can be used without causing distortion of the zones varies with the agarose concentration, but can easily be determined by introducing a zone containing a dye.

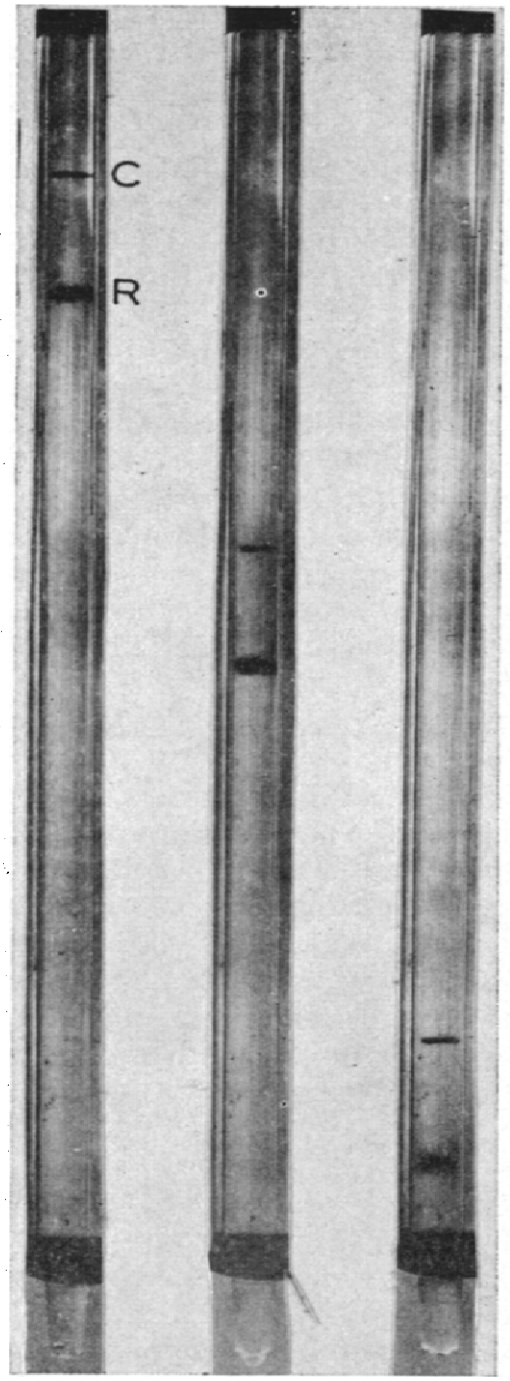
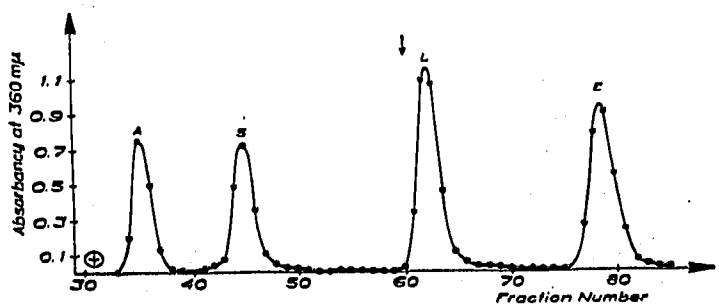


Fig. 5. Photographs, illustrating that the emptying procedure does not cause a detectable broadening of the zones. The zones C and R contain *Chlorella pyrenoidosa* and R-phycoerythrin, respectively. The column, of dimensions 0.9 × 50 cm, was emptied at a rate of 10 ml/h.

a



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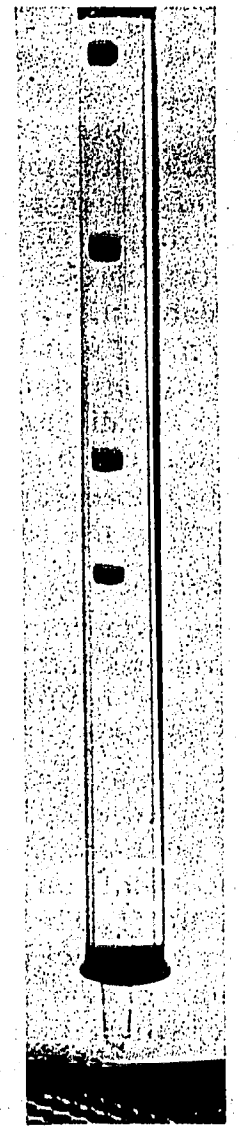

Fig. 6. (a) Electrophoresis in a 0.16% agarose suspension of a mixture of DNP-aspartic acid (A), DNP-serine (S) and ε-DNP-lysine (L). Electrophoresis tube dimensions: 0.9 × 50 cm. Buffer: sodium acetate, pH 4.85, ionic strength 0.01, + 0.005 *M* calcium chloride. Sample: 0.4 ml agarose suspension containing 0.2 mg of each DNP-amino acid. Current: 6 mA. Duration: 3 h. Emptying rate: 10 ml/h. The place for application of the sample is indicated by an arrow. As a neutral marker, DNP-ethanolamine (E) was applied at fraction 76. (b) A photograph of the separation in Fig. 6a. The column is of an older type with an outer "shunt", which has to be detached before the empyting and is therefore not seen in the photograph.

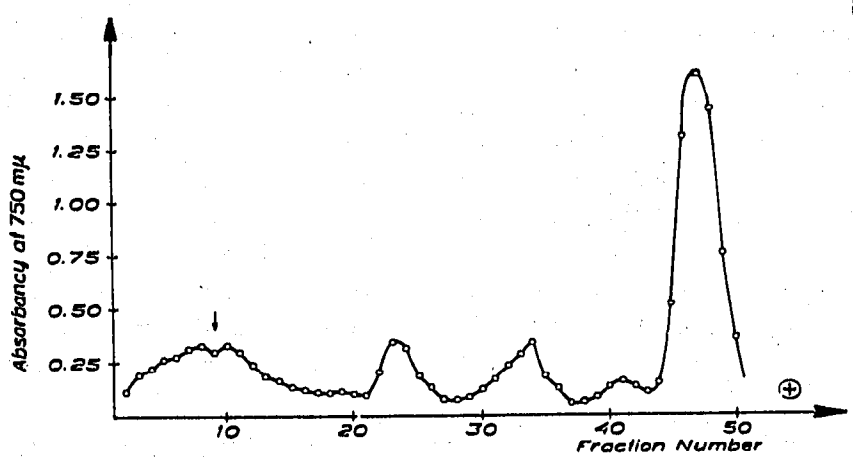

Fig. 7. Electrophoresis in a 0.17% agarose suspension of normal human serum. Electrophoresis tube dimensions: 0.5 × 55 cm. Buffer: sodium veronal, pH 8.6, ionic strength 0.05. Sample: 75 μl serum + 75 μl agarose suspension. Current: 5.5 mA. Duration: 17 h. The column was emptied by sucking out 1-cm fractions. The arrow at fraction number 9 indicates the position of the starting zone. The protein contents were determined by absorption measurements at 750 mμ according to the method developed by FOLIN–LOWRY[5].

2.0 × 85 cm (Fig. 3a). About 2 ml dialyzed normal human serum was diluted (1:1) with the agarose suspension and applied 22 cm from the top of the cooling mantle. The current was adjusted to 50 mA. The experiment was interrupted after 62 h when the yellow albumin zone was 20 cm from the bottom of the cooling mantle. The column was emptied at a pump rate of 60 ml/h (the technique is as described for wide columns). 100 μl of each 2-ml fraction was used for the determination of the protein concentration according to the FOLIN–LOWRY method[5]. The six peaks (Fig. 8) were analyzed by paper electrophoresis, and, as expected, they contained prealbumin, albumin, α_1-globulin, α_2-globulin, β-globulin, and γ-globulin. In these analyses, a zone-sharpening method[12] was used, which allows dilute protein samples to be applied directly to the paper without previous concentration (owing to electroendosmosis the γ-globulin did not reach the dialysis bag and therefore could not be zone sharpened by this method). The result is given in Fig. 9. The spot corresponding to prealbumin was poorly reproduced in the photograph and has therefore been omitted. The paper electrophoresis was conducted in the same buffer as the agarose electrophoresis.

Electrophoresis of subcellular particles

These experiments were performed before the agarose was introduced as an anti-convection agent and before the electrophoresis apparatus was fitted with a "shunt". The agar* column was therefore compressed during the electrophoresis (the position of the starting zone is therefore not indicated in Figs. 10 and 11). Comparatively few cracks were, however, observed, which may be due to the fact that the buffer used contained magnesium ions**.

(*a*) *Electrophoresis of a microsome preparation from rat brain.* Some preliminary experiments on microsomes from rat brain were carried out in collaboration with DR. TOSCHI***. The microsomes were prepared as described by him[13]. About 0.75 ml of the sample, which contained around 5 mg protein per ml, was mixed with an equal volume of agar suspension and applied about 15 cm from the top of the electrophoresis tube, which had the dimensions 0.9 × 60 cm. The temperature of the circulating cooling water was +0.5°. The electrophoresis was conducted for 12 h at 10 mA in a sodium borate buffer, pH 8.2, ionic strength 0.03, containing 0.0083 *M* sodium sulfate and 0.001 *M* magnesium sulfate. The column was emptied as described previously for intermediate columns. Spectrophotometric measurements at 260 mμ in a 1-cm cuvette of 1.5-ml fractions gave the electropherogram in Fig. 10. The ribonucleic acid (RNA) and the protein contents were determined in the two main fractions A and B[13]. Although variable results were obtained with different microsome preparations, the RNA concentration (on protein basis) was always higher in the faster migrating zone A. The highest value observed in this zone was about 250 μg RNA per mg protein, while zone B in the same experiment contained about 20 μg RNA per mg protein. As shown by HANZON AND TOSCHI[14] with the aid of electron microscopy, brain microsomes consist of two submicroscopic structures, vesicles formed by thin membranes and small dense

* 0.14% suspensions of Difco Noble Agar were used.

** When an electrophoresis is performed at potential gradients as high as 40 V/cm, especially in buffers of low ionic strength, cracks may sometimes occur also in agarose suspensions. In such cases the tendency for crack formation can be decreased if a plug N of a gel of agarose or polyacrylamide is used instead of a dialysis membrane D (Fig. 1) and if magnesium ions are added to the buffer, for instance in the form of magnesium chloride, to a concentration of 0.004 *M* (for preparation of these plugs, see the section "Narrow columns").

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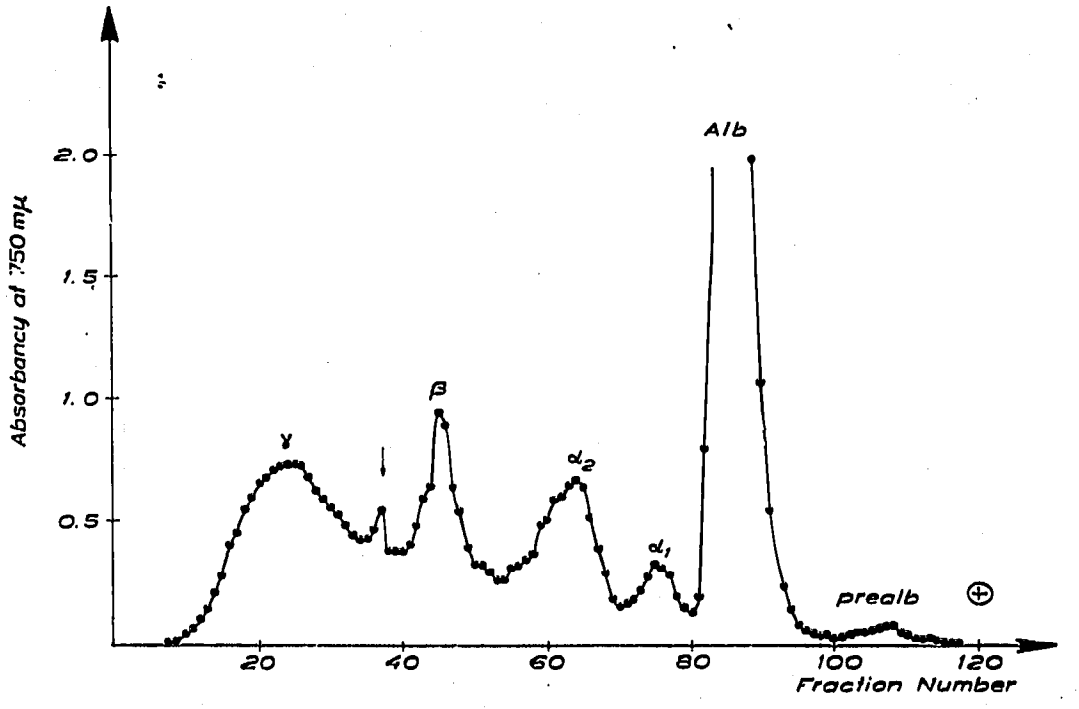

Fig. 8. Electrophoresis of normal human serum. Agarose concentration and buffer composition were the same as those given in the legend to Fig. 7. Electrophoresis tube dimensions: 2.0 × 85 cm. Sample: 2 ml serum + 2 ml agarose suspension. Current: 50 mA. Duration: 62 h. Emptying rate: 60 ml/h. The arrow at fraction number 37 indicates the position of the starting zone.

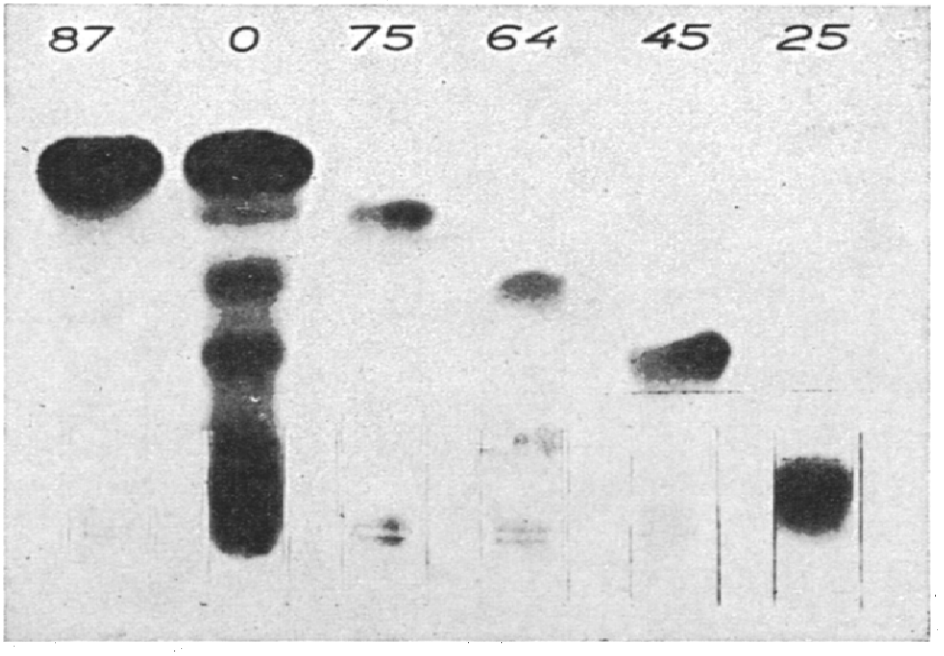

Fig. 9. Paper electrophoresis of the material in the main peaks in Fig. 8, *i.e.* fractions 25, 45, 64, 75 and 87. The pattern indicated by O was obtained from unfractionated serum. By using a zone-sharpening method[12] no preconcentration of the fractions was necessary, except for γ-globulin.

particles, RNA being associated with the particles. Electronmicroscopic examination of samples from the zones A and B showed a definite separation of the particles, which collected in zone A in high concentration.

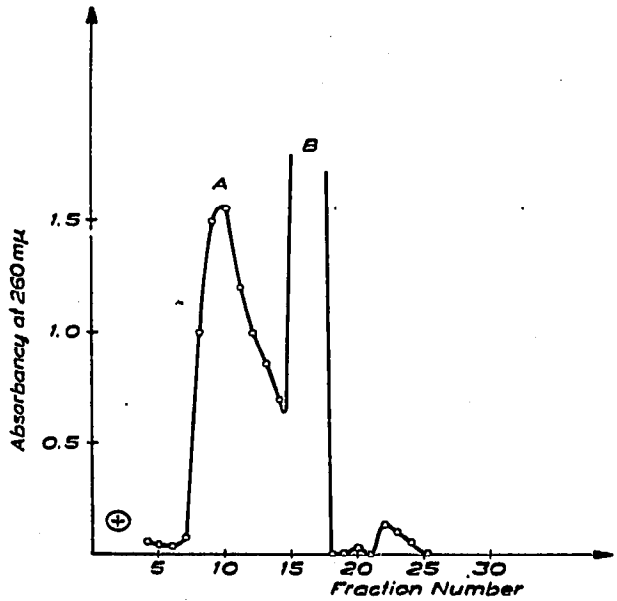

Fig. 10. Electrophoresis in a 0.14% agar suspension of a microsome preparation from rat brain. Electrophoresis tube dimensions: 0.9 × 60 cm. Buffer: sodium borate, pH 8.2, ionic strength 0.03, + 0.0083 *M* sodium sulfate + 0.001 *M* magnesium sulfate. Sample: 0.75 ml of the microsome preparation + 0.75 ml agar suspension. Current: 10 mA. Duration: 12 h. Emptying rate: 10 ml/h. The fastest migrating zone A has a much higher RNA concentration (on protein basis) than zone B. The dense particles[14] were highly enriched in zone A.

(*b*) *Electrophoresis of a microsome preparation from baker's yeast.* This experiment was carried out in collaboration with Dr. LEVIN in this Institute. The microsomes were prepared essentially as described by CHAO AND SCHACHMAN[15]. The microsomes were suspended in a solution, 0.0125 *M* with respect to potassium phosphate, pH 7.45, and 10^{-3} *M* with respect to magnesium sulfate. For preparation of an agar suspension of this ionic composition, the magnesium sulfate must be added to the phosphate buffer after the agar suspension has cooled to prevent precipitation. No agar suspension was added to the sample and therefore it was not layered but injected into the agar column as described under "Application of the sample". The volume of the microsome solution was about 2.5 ml and it was injected 15 cm from the top of the cooling mantle. The dimensions of the electrophoresis tube were 2.0 × 85 cm. The experiment was performed at 20 mA for 23 h. After some hours' electrophoresis three opalescent zones could be observed. The fastest of these, however, became gradually so faint that it could no longer be detected. The column was emptied at a rate of 2 ml/min, with the aid of the drainage device shown in Fig. 3 b. The different fractions were analyzed by absorption measurements at 260 and 280 mμ in a 1-cm cuvette. An estimation of the protein contents were made also by the FOLIN–LOWRY method[5]. The result of these analyses is given in the electropherogram in Fig. 11, which shows that the main peak I is followed by a peak II, somewhat richer in protein. The slowest migrating zone, IV, was most opalescent; its protein contents must be very low to judge from the Folin-curve.

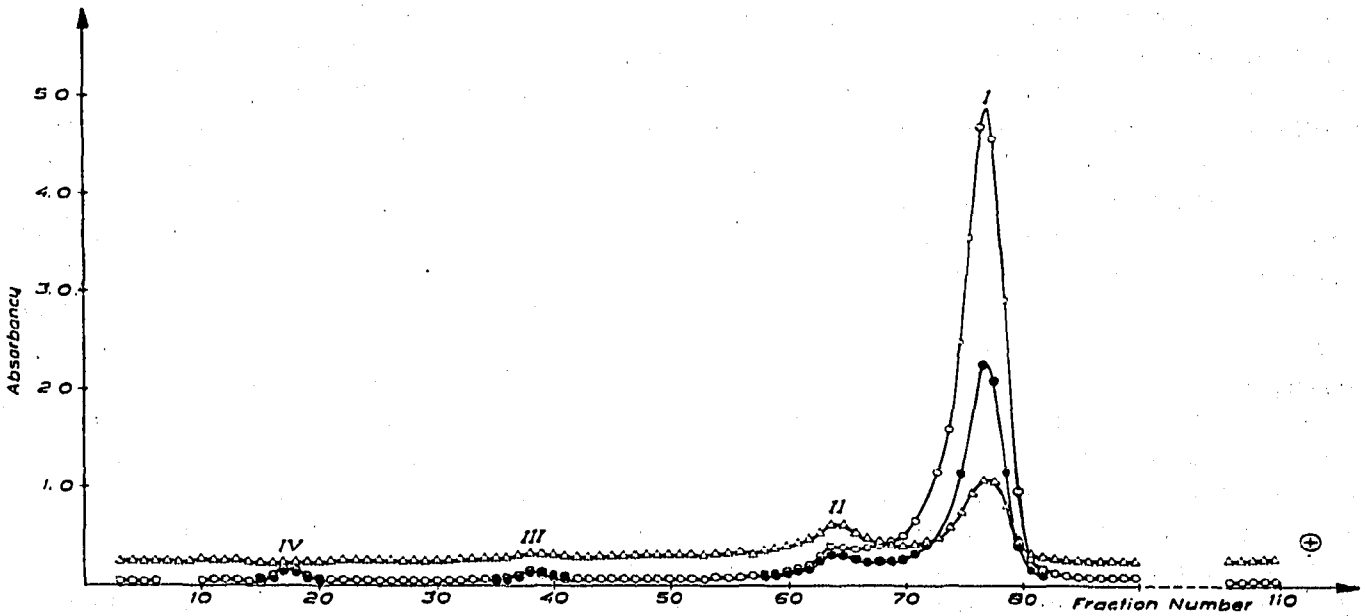

Fig. 11. Electrophoresis in a 0.14 % agar suspension of a microsome preparation from baker's yeast. Electrophoresis tube dimensions: 2.0 × 85 cm. Buffer: 0.0125 *M* potassium phosphate, pH 7.45, + 0.001 *M* magnesium sulfate. Sample: 2.5 ml of the microsome preparation. Current: 20 mA. Duration: 23 h. Emptying rate: 120 ml/h. Open and solid circles correspond to absorption measurements at 260 and 280 mμ, respectively. The protein contents (triangles) were determined at 750 mμ according to the method of FOLIN–LOWRY[5]. Some microsome preparations contain more material in peaks II, III and IV, and sometimes a smaller peak is obtained in front of peak I.

DISCUSSION

Agarose suspensions provide good stabilization against convection even at concentrations as low as 0.15 %. It is therefore not surprising that the electrophoretic migration velocities of proteins in these suspensions are similar to those in free solution (Table I). Each preparative agarose electrophoresis experiment can therefore be utilized for approximate determinations of mobilities as well, provided a neutral reference substance is used to correct for electroendosmosis[8], which may be rather pronounced (Figs. 6 a and 7). The fact that the "sieving effect" is very low in agarose suspensions is in accordance with the finding by ACKERS AND STEERE[17] that in a 0.15 % agar gel the diffusion coefficients of hemoglobin and southern bean mosaic virus agree within a few percent with the values obtained in free solution. The pore size of the gel matrix thus allows migration of much larger cell particles than the microsomes used in the above experiments (Figs. 10 and 11). It should be pointed out, however, that when the particle size is extended to whole cells, like bacteria, the sample remains at the place of application.

Electrophoresis in agarose suspensions often gives very sharp separations. Fig. 8 may serve as an example. As seen, α_1-globulin in human serum can easily be isolated from albumin, a separation not so easily obtained in preparative zone electrophoresis. Another example is Fig. 6 in ref. 2, which shows that the major part of the enzyme activities after the run was found in a volume only three times larger than that of the applied sample. The creation of such narrow zones is favored by the homogeneity of the agarose columns, as well as by the extremely low tendency to adsorption[1]. A special advantage is also the fact that the emptying procedure does not cause a

detectable distortion of the zones (Fig. 5), as suspensions of agarcse move as a rigid body or piston when forced through the electrophoresis tube ("plug flow").

Before starting experiments on a large column it may save time and material to make preliminary experiments on a small column in order to ascertain which buffer, pH, and ionic strength etc. will give the best resolution. This requires the result obtained with the small column to be similar to that obtained with the large one. That this is the case when agarose suspensions are used as the supporting medium is evident from a comparison between Figs. 7 and 8.

As the agarose columns are semi-transparent, the highest potential gradient that may be used without the zones being distorted can in each experiment be determined easily by observing the shape of the sample zone. If this is not visually detectable, a zone of colored protein is introduced (for this purpose low molecular weight substances should not be used, because they have a strong diffusion which will be superimposed on the above distortion and make its detection more difficult). When the instability of the sample requires short duration of the experiment one may be forced to use an extremely high potential gradient. The resulting curvature of the zones gives rise to a decrease in the resolution. If necessary this can, however, be considerably increased by forcing agarose suspension into the electrophoresis tube in a direction opposite to that of the migration of the zones. If such a hydrodynamic compensation is made at a suitable rate a curved zone can be restored to its original flat shape.

As further examples of proteins being purified by the electrophoresis method described in this paper, uridine kinase from Ehrlich ascites tumor[18], uridine and deoxyuridine phosphorylases from the same source[19], and human growth hormone[20] can be mentioned.

We have earlier pointed out that agarose has some advantages over agar as an anticonvection agent[1,21]. This has now been confirmed by GHEŢIE AND MOŢET-GRIGORAŞ[22] who found that in electrophoresis of human serum, agarose gels give about twice as many zones as agar gels. The same authors also claim that a more easily interpretable pattern of immunoelectrophoretic precipitation lines is obtained with agarose.

A great number of electrophoresis methods are now available for purification of substances of biological origin. It is beyond the scope of this paper to discuss all their advantages and disadvantages, but a brief comparison might be appropriate.

An ideal anticonvection agent should not, in general, show any tendency to adsorb the substances to be separated. In this respect columns of agarose suspensions are preferable to those of starch grains or cellulose powder. Plastic powders, for instance Pevikon, are also characterized by a low adsorption, but unfortunately they are not particularly efficient as anticonvection media[2]. The broadening of a zone during the elution or emptying procedure is of great interest when discussing different electrophoresis methods. Displacement of the zones from a column by a hydrodynamic buffer flow involves an inevitable increase in their width. As pointed out previously, however, the emptying technique used for agarose columns causes a negligible broadening of the zones. Another factor that must be taken into account in these comparisons is the degree of contamination of the bed material with the isolated fractions. In this respect Pevikon is superior to agarose (as mentioned, the concentration of agarose in the supernatant after centrifugation is about 0.007 %); cellulose and starch take an intermediate place. In some cases the presence of agarose is a disturbing factor. Thus,

in determinations of the carbohydrate content of a protein, agarose electrophoresis should not be used at the last step in a series of purification methods. The traces of agarose in the supernatant can, of course, be removed by electrophoresis or chromatography on inert columns (owing to the neutral character of agarose it is easy to establish the experimental conditions for its removing).

The bed materials discussed here give electropherograms which resemble those obtained in free electrophoresis. During recent years gels of starch and polyacrylamide have been introduced, which, however, give electropherograms of a totally different appearance owing to the "molecular-sieving" action of these gels. If a sample is submitted to two consecutive electrophoresis runs, one in a medium without and the other in a medium with "molecular-sieving" properties, a high grade of purification can be expected. Electrophoretic "molecular-sieving" has frequently been used for analytical purposes. In its present form it can be utilized only for preparative purposes on a mg scale[23]. The difficulty of eluting the zones without distortion increases with the cross-section area of the gel. If electrophoretic "molecular-sieving" is to be developed as a large scale preparative method the elution technique must be refined.

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SUMMARY

Suspensions of gel particles of agarose can be used as supporting media for electrophoresis on an analytical as well as a preparative scale for purification of low molecular weight substances, proteins, and also of subcellular particles, such as microsomes. Particular advantages are that the adsorption to the agarose of the substances to be separated is extremely low and that the columns can be emptied with negligible distortion of the zones. The agarose is removed by centrifugation. As the agarose concentration can be kept as low as 0.16%, the suspensions are semitransparent, and so colored and opalescent zones can be detected comparatively easily. The electrophoretic migration velocities of proteins in these suspensions are similar to those in free solution.

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