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Electrophoresis in a Density Gradient

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In the method of zone electrophoresis a means for the stabilization of the medium against convection has to be provided. That a density gradient of sucrose might be suitable for this purpose was suggested by Consden, Gordon & Martin (1946). Since then several attempts, discussed by Svensson, Hagdahl & Lerner (1957), have been made to achieve separations on this principle. In such systems it is comparatively easy to obtain good separations of substances if they are present in such small amounts as not to contribute significantly to the density of the initial solution, or if they are of approximately the same molecular size as the substance used for making the gradient. These conditions can be met quite satisfactorily with dyestuffs in a sucrose gradient, but not so easily with proteins. With large molecules, instability of the initial zone develops rapidly, even though its density has been adjusted so that it is slightly less than that of the solution on which it rests. This happens because the decrease in density due to diffusion of large molecules out of the zone is more than compensated by the more rapid diffusion of sucrose into the zone. As a result, the zone becomes relatively dense and droplets are formed. Fortunately this difficulty has now been overcome by Svensson *et al.* (1957), who have shown that droplet formation in the initial zone does not occur if an appropriate amount of a dense, rapidly diffusing substance such as potassium bromide is present in the protein solution. Most of Svensson's work was carried out either with dyestuffs (Svensson & Valmet, 1955) or with normal or pathological sera (Svensson *et al.* 1957). The aim of the present work has been to examine the reproducibility and completeness of the separation of proteins in a modification of the apparatus used by Svensson *et al.* (1957). For this

purpose human albumin and artificial mixtures of human albumin and haemoglobin have been used.

Complete separation of many of the proteins of human and animal sera can, of course, be achieved readily by the method of zone electrophoresis described by Porath (1954). Probably the most serious disadvantage of this method is the adsorption of traces of the proteins on the cellulose matrix of the column. Evidently the sucrose-gradient column avoids all difficulties due to adsorption, except on the glass of the apparatus. Since the possibility of adsorption of virus particles on a solid matrix cannot be ignored, it is interesting to note that Cramer, Lerner & Polson (1957) have recently employed sucrose-gradient electrophoresis for a problem of this kind. We report here an application to the slow-reacting substance A of Brocklehurst (1955, 1956), which possesses the property of stimulating guinea-pig gut.

EXPERIMENTAL

The apparatus (Fig. 1), basically the same as that of Svensson *et al.* (1957), incorporates some novel features. It was constructed with all-glass joints, held together by springs. The joints at the top of the electrode vessels were inverted to eliminate accumulation of large gas bubbles, which otherwise were periodically detached during electrophoresis, producing slight but undesirable movement of the liquid. Filling of the gradient tube was made automatic. The liquid from the mixer was fed through a long capillary tube which was held in contact with the wall of the column about an inch above the meniscus. This capillary was raised intermittently by means of a screwed-rod mechanism actuated by two contacts attached to the capillary tube and touching the liquid surface. Virtually no current passed between the contacts, since the control was exercised through a sensitive electronic relay.

With sucrose columns, sections 2 and 4 (Fig. 1) were first filled with plain buffer. Next the capillary tube 7 was

filled with buffer containing 50% (w/v) of sucrose (by suction), which was also used to fill vessel 8 to a height of several inches, and column 6 to a few centimetres below the top of the capillary. The rest of vessel 8 was filled with plain buffer. After the gradient had been produced in 6, the apparatus was filled to the top with plain buffer, and thus completely filled with liquid. Two or three 10 ml. samples were then withdrawn from tap 7 by siphoning buffer in through tap 3. The sample, its density adjusted with the help of an auxiliary oil gradient, was siphoned in via tap 7.

Fig. 1. Diagram of apparatus. Enlarged inset shows exit holes for gas at top of electrode vessels.

The potassium bromide concentration (w/v) was made equal to that of the protein, as this condition prevented droplet formation. Generally approx. 3 ml. of sample was introduced in 20–30 min., producing a zone about 5 mm. high. To minimize movement of the initial zone, levelling was carried out by adjusting the heights of the menisci in the narrow tubes at the top of 1 and 9 before these taps were opened. The difference in the levels of the menisci, to compensate for the gradient column, was usually about 15 and 5 mm. for sucrose and ethanol respectively. The meniscus on each side was then lowered by the same distance (with the appropriate tap open) in order to bring the levels to wider regions of 1 and 9 before taps 1 and 9 were both finally opened.

With ethanol gradients, the whole apparatus was filled with plain buffer, except for the column, and for the top of vessel 4 and the region 5, which contained buffer mixed with ethanol. Theoretically it would be preferable for the aqueous buffer in the mixer to be free of air, but in practice most of the air expelled on mixing with the 50% (w/v) ethanol is eliminated as the solution flows out of the capillary tube feeding in the gradient. The auxiliary oil gradient proved unsuitable, owing to loss of ethanol from the drops to the column. Densities were, therefore, adjusted by means of floats, each of which consisted of a small piece of polythene sheet transfixed by a short length of Nichrome wire (the particular polythene used gave floats of about 8 mm. diameter each carrying 2–3 mm. of 0.27 mm. diameter wire). The mean density of these floats could be increased when necessary by clipping off small sections of the polythene. It was essential, of course, to prevent air bubbles sticking to the floats. The potential difference applied to the apparatus, 450–550 v, produced an initial current of 20–27 ma, which dropped by 20–30% during 24 hr. and more rapidly subsequently, unless the buffer in the left-hand electrode vessel was renewed. When this was done, the final drop over 48 hr. was about 30% of the initial value.

At the conclusion of the run, 2–3 ml. fractions were run out at the rate of 1–2 ml./min. These were used for measurements of visible light-absorption at 540 m μ (with haemoglobin) or ultraviolet absorption at 279 m μ (with albumin and insulin) in the Beckman DU spectrophotometer. In the most accurate work, corrections were applied for the absorption of the sucrose (or possibly impurities in it) after the sucrose concentration in various tubes had been estimated by means of dipping refractometer measurements on samples diluted by weight. The shape of the sucrose gradient was nearly, but not quite, linear at the end of the run. The A.R. sucrose used in all experiments gave blank readings approximately 50% of those of ordinary sucrose. In sucrose gradients, 0.025 M-borax (pH 9.2) was used, as recommended by Svensson *et al.* (1957). In ethanol gradients 0.025 M-ammonium acetate was used, adjusted by means of ammonia to pH 8 or 9. Neither of these buffers absorbed significantly at 279 m μ .

The experiments illustrated by Figs. 2–4 were done with human albumin (sample A) used in other work (Charlwood, 1954). Another sample used was prepared by column electrophoresis (Porath, 1954). Haemoglobin was prepared from washed human red cells by haemolysis with water and toluene, followed by high-speed centrifuging to remove the stroma. The crystalline zinc insulin (batch number 9333/1404) was supplied by Burroughs Wellcome and Co.

Iodinations were carried out by the method of McFarlane (1956), to the extent of a mean substitution of not more than 1 atom of total iodine/mol. In most cases the albumin was subsequently diluted with several times its weight of untreated albumin. Radioactivities of iodinated samples were measured on weighed samples in a scintillation counter, and corrected to a volume basis. Error due to the counter was never more than 3% and generally better than 1%.

The slow-reacting substance was prepared from perfused guinea-pig lungs and partially purified by Mr W. E. Brocklehurst as described previously (Brocklehurst, 1955). This material, of unknown composition, was estimated by its ability to cause contraction of the isolated guinea-pig ileum. Gradient electrophoresis experiments were done with samples containing 2000–9000 effective doses, at which levels the weight of the material introduced was small (<5 mg.).

RESULTS

These have mostly been plotted as curves, although strictly histograms should be used. The reproducibility of the technique is shown by the results of duplicate runs on human albumin (Fig. 2). The points shown, one set obtained with the use of a fraction collector, the other by manual collection of fractions, all lie virtually on the same curve. They are uncorrected for the solvent background, but the magnitudes of these corrections are shown by the triangles in Fig. 2. It is thus clear that tubes before number 25 contained no detectable protein.

Fig. 3 shows results of an experiment designed to study the quality of separations. The two proteins haemoglobin and albumin were located by entirely

independent methods, the former by absorption in the visible region and the other by radioactivity measurements of the ^{131}I incorporated in it. Evidence of freedom from mutual contamination was obtained by reciprocal measurements, i.e. no radioactivity could be found in the haemoglobin, nor light-absorption at $540\text{ m}\mu$ in the albumin solutions.

Fig. 4 shows the distribution of optical density and radioactivity in a typical albumin run. The peak of activity in different experiments moved consistently just ahead of that of optical density. This is shown clearly by the curve which gives the specific activity of different fractions, expressed arbitrarily as the ratio of counts/min./ml. to optical density. The specific activity increased steadily from tubes 22 to 28, but then fell again,

Fig. 3. Separation of 20 mg. of haemoglobin (O) from 30 mg. of human ^{131}I albumin (●) after 40 hr. electrophoresis in a sucrose gradient containing 0.025 M-borax (pH 9.2). Current 23–17 mA. The haemoglobin was located by absorption at $540\text{ m}\mu$, and the albumin by its radioactivity.

Fig. 2. Ultraviolet-absorption curve obtained after electrophoresis of 30 mg. of human albumin for 45 hr. in a sucrose gradient containing 0.025 M-borax (pH 9.2). Current 23–17 mA. Fractions collected manually are shown by filled circles, those collected automatically in a duplicate run by open circles. Triangles denote absorption arising from the batch of sucrose used.

Fig. 4. Ultraviolet-absorption curve (●) obtained after electrophoresis for 40 hr. of 45 mg. of human ^{131}I -albumin, in a sucrose gradient containing 0.025 M-borax (pH 9.2). Current 22–16 mA. Specific radioactivities of the fractions (○) and of the starting material (arrows) are also shown.

behaviour which is referred to in the Discussion. When the contents of tube 26 were mixed with a small volume of concentrated untreated albumin and re-run, the curves obtained were still very similar. In a repetition of this experiment, at the conclusion of the run the contents of the gradient column were expressed through a capillary tube at the top of the column. The last centimetre of this tube, which extended approximately 3 cm. outside the gradient tube, was curved downwards to facilitate collection of fractions while minimizing mixing. The curves obtained were still of the same type as before.

Curves obtained after 1- and 2-day runs on insulin in ammonium acetate buffer and ethanol are shown in Fig. 5. The main feature to note is the shoulder on the trailing edge of the peak. The spread was somewhat greater than in an experiment with insulin (not shown) in the sucrose gradient.

Electrophoresis of the slow-reacting substance A in a sucrose gradient was carried out for 24 hr., after which the 3.0 ml. fractions obtained from the column were tested biologically by Mr Brocklehurst with the results shown in Fig. 6. More than 70% of the activity applied to the column was accounted for in tubes 32-37. As it was not possible to separate the active material from the sucrose by dialysis, experiments were carried out in the volatile system used for insulin, but at a lower pH (8.0). A typical set of results is illustrated in Fig. 7, which shows that a reasonably sharp peak is obtainable under these conditions. A minor distribution of active material, which differed qualitatively in biological behaviour from the

major component, appeared moving more slowly than the main peak. There is reason to believe that the minor component may be a breakdown product of the other.

Fig. 6. Histogram showing biological activity of fractions obtained after electrophoresis of a sample of slow-reacting substance A for 22 hr. in a sucrose gradient containing phosphate buffer (pH 8.0, 0.05 I). Current 24-20 mA. Arrows show limit of sensitivity of biological assay.

Fig. 5. Ultraviolet-absorption curves obtained after electrophoresis of 50 mg. of insulin in an ethanol gradient containing 0.025 M-ammonium acetate adjusted to pH 9.0 with ammonia: (●) after 16 hr. (current 21-20 mA); (Δ) after 44 hr. (current 16-14 mA).

Fig. 7. Histogram showing biological activity of fractions obtained after electrophoresis of a sample of slow-reacting substance A for 17.5 hr. in an ethanol gradient containing 0.025 M-ammonium acetate adjusted to pH 8.0 with ammonia. Fractions from minor peak showed a modified gut response (see text).

DISCUSSION

Some of the advantages of this type of electrophoresis have already been mentioned in the introduction. It seems probable, however, that the usefulness of the method will depend largely both on the reproducibility of the separations which can be obtained and on the shapes of bands after separation, with particular reference to degree of resolution. The results recently published by Berg & Beeler (1958) show an apparently good resolution for human serum and plasma, although, as they themselves state, the method of protein estimation used must have led to an exaggeration of the albumin peaks. As appears in Fig. 2, very precise reproducibility from experiment to experiment has been obtained with human serum albumin. The shape of the band of human albumin after electrophoresis has been compared with that calculated for diffusion alone after the same time. Although the latter, as must be expected, is considerably sharper, the greater spread of the former is within acceptable limits, as 84 % of the albumin in each experiment (Fig. 2) is accounted for in five tubes. A slight asymmetry, consisting mainly of a more diffuse leading edge, is, however, detectable. Since the albumin used was of known electrophoretic homogeneity (Charlwood, 1954), this phenomenon requires explanation. Several possibilities have been considered, including 'tailing', and viscosity gradient in the tube. It seemed likely that, if the gradient were allowed to run out too fast, 'tailing' would result from the increased drag experienced by regions of the very viscous sucrose solution nearer to the walls of the main tube. In the experiment with the apparatus specially modified to permit running out from a capillary fixed at the top of the gradient, the usual band shape was found. Since reversal of the shape of the band did not take place, it appears that tailing cannot be of more than very minor importance at the rates of running out used in the present work. It seems likely that effects of this kind will be even less important when the gradient has been made from ethanol instead of from sucrose, because of the much lower viscosities of solutions of the former substance. In the present work both ethanol and sucrose gradients have been run out at approximately 1.5 ml./min. This is considerably slower than the rate suggested for ethanol gradients by Svensson & Valmet (1955). The marked asymmetry of certain components in the diagrams of human serum (Svensson *et al.* 1957) may perhaps have resulted from unduly fast running out, but unfortunately these authors did not state the rate at which their sucrose gradients were run out.

When trace-iodinated human serum albumin was used, the peak of radioactivity moved slightly

further up the tube than did the peak of optical density. A leading edge showing higher radioactivity than that of the main band has previously been reported by Gabrieli, Goulian, Kinersly & Collet (1954), who subjected commercially iodinated (Abbott) human serum albumin to electrophoresis on paper. On the other hand, trace-iodinated albumins (Cohen & Shamroth, 1958) do not show this effect by paper electrophoresis. The specific activities of the albumin fractions (Fig. 4) showed a maximum, instead of rising steadily as might be expected if, on the average, the iodinated molecules had a slightly higher mobility than those without iodine. This behaviour was reproduced with the albumin from the Porath column and was not therefore something peculiar to the other batch. Further, in the experiment mentioned above, in which fractions were taken out from the top of the tube, the curve of specific activity again showed a maximum and, indeed, was very similar to that obtained by running out from the bottom. This effect, which is capable of several interpretations, apparently does not derive from the conditions of running out, but must be related to the relative rates of movement of the protein species present. Evidently the specific activity measurements provide a refined indication of very slight degrees of separation.

The main purpose of the experiments with insulin was to ascertain whether a substance of large molecular weight could be made to move reproducibly and without excessive spreading in an ethanol gradient. The degree to which this has been achieved is shown in Fig. 5, which gives the band shapes obtained after 16 hr. and in a second experiment under the same conditions except that it extended to 44 hr. Rather similar unsymmetrical patterns have been reported for this protein by Timasheff, Brown & Kirkwood (1953), who used barbiturate buffer at pH 8.6 in the Tiselius apparatus. On the other hand, the existence of a boundary of lower mobility could not be confirmed in the present work when insulin was examined at pH 9.2 in a sucrose gradient. Possibly the boundary shape first noted by Timasheff *et al.* (1953) is due to an association-dissociation reaction which is suppressed in concentrated sucrose solutions.

The experiments with albumin-haemoglobin mixtures (see Fig. 3), and other evidence in this work, indicate that electrophoresis in a density gradient is capable of providing good resolution with moderate loads of protein. Satisfactory runs have been made with 250 mg., but there must be a limit to the quantities which can be separated by this method; for instance, it could hardly be expected to operate on the scale which can be used for zone electrophoresis in a solid matrix (Porath & Gedin, 1957). On the other hand, the absence of a

matrix has certain advantages. Since adsorption is ruled out, choice of the inert material in the column becomes much simpler. The requirements for the material from which the gradient is formed are that it should be a non-electrolyte, unable to react with the substances being investigated and, in preparative work, easily separable from these substances. In the present work only sucrose and ethanol have been used for the gradients, but for other problems different materials might well be more suitable. The outstanding advantage of the gradient method, due to the relatively small surface area of solid exposed to the samples, has been illustrated in the present work by the experiments with slow-reacting substance A. Thus, whereas the ease with which this material is adsorbed has prevented useful electrophoresis in other zone systems, adequately sharp bands have now been obtained both in sucrose and in ethanol gradients. Thus it is probable that this type of zone electrophoresis will be of increasing usefulness in the separation of biologically active substances which are readily adsorbed on the matrix materials used for zone electrophoresis.

SUMMARY

1. A density-gradient electrophoresis apparatus somewhat modified from that designed by Svensson is described. Experiments have been carried out in gradients made from ethanol and from sucrose.

2. The reproducibility of movement and shape of bands of human albumin and the completeness of separation of this protein from a band of haemoglobin have been studied in the sucrose gradient.

3. The specific activities of the fractions thus obtained from human plasma albumin trace-iodinated with ^{131}I have been found to vary con-

siderably and to show a maximum just preceding the maximum protein concentration as measured by ultraviolet-light absorption.

4. Insulin has been caused to migrate in both the sucrose and the ethanol gradients; in the latter the protein concentration curve possessed a shoulder.

5. Suitable conditions have been found for the electrophoresis of slow-reacting substance A, a substance causing constriction of the guinea-pig ileum.

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Assimilation of Carbon Dioxide by Yeasts

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The formation of carbon dioxide during glucose oxidation or fermentation by yeasts is well established. The fixation of carbon dioxide is less well known, though its existence is supported by the following evidence: (1) Carbon dioxide is necessary for the growth of yeasts (Rockwell & Highberger, 1927) and the synthesis of succinic acid by *Saccharomyces cerevisiae* (baker's yeast)

(Kleinzeller, 1941). (2) Both carbon dioxide and dicarboxylic acids promote substrate oxidation by baker's yeast (Sperber & Runnström, 1939; Stoppani, 1951) but their actions are not additive. The replacement effect indicates that carbon dioxide is incorporated into dicarboxylic acids, which are the true stimulants of substrate oxidation. (3) During pyruvate oxidation in a medium