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Density Gradient Electrophoresis as a New Tool in Virology*

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Introduction. Viruses in the native state are usually present in extremely low weight concentrations, together with large amounts of foreign material, mostly proteins. In crude preparations, it is thus in general impossible to detect or measure the virus by physical or chemical means. The purification procedure, therefore, has to be controlled by infectivity assays, which have a sentitivity 10^4 to 10^8 times as high as, for instance, analysis by ultraviolet light absorption.

Exceptions to the above are some plant viruses, for which there is strong evidence that they have been visualized in crude extracts by physical methods[1]. However, this has not been proved by biological tests.

The isolation of trace amounts of virus from huge quantities of inactive contaminants offers, of course, great difficulties, especially as many viruses are extremely unstable and easily lose their activity under the influence of physical and chemical treatment. The fact that viruses are the very heaviest molecules hitherto known, with molecular weights in the range 10^8, together with the availability during the last few decades of preparative high-speed centrifuges, has greatly facilitated this task. The technique of high-speed differential centrifugation has been extremely important for purification of viruses. In general, microscopic and larger particles of tissue debris are first thrown down by a centrifugation at a comparatively low speed and discarded. The virus is then brought to sediment completely in an extended run at high speed, leaving ordinary proteinous material in the supernatant. The procedure is repeated. With reference to the title of this article, it is interesting to note the increasing importance of high-speed *zonal* centrifugation in density gradients made up by a radially increasing sucrose concentration in the centrifuge tubes. This technique was introduced by BRAKKE[2,3] and was used by him for purification of plant viruses.

Other principles that have been important for purification of viruses are those common to ordinary protein chemistry: salting out with ammonium sulphate, isoelectric precipitation, precipitation by other agents, adsorption and elution. Chromatography on ion exchangers has been used occasionally[4].

Concerning the physico-chemical characterization of purified viruses, especially of preparations that can be regarded as almost 100% pure, sedimentation, diffusion, and electron microscopy are the most important methods. They give together exact information of size, shape and molecular weight of the particles. Light scattering is another useful aid for estimating molecular data[5].

Stability characteristics are of great interest. Like other biologically active substances, the viruses are inactivated by exposure to extreme pH, to various chemical agents, to physical treatment, or simply by storage. Such characterization does not require purified preparations, but they do require infectivity tests.

Electrochemically, a virus is best characterized by its isoelectric point (which may depend on the salt concentration) and by a curve relating its charge, its proton-binding capacity, its electro-kinetic potential, or its mobility with pH. The protonbinding capacity can only be obtained by a direct potentiometric titration of a pure virus sample. The isoelectric point and the pH-mobility curve can be obtained by a series of electrophoresis experiments with preparations sufficiently pure to make it possible to identify a certain peak in the electrophoresis diagram with the active virus. Rough estimates of mobility data may be obtained from crude extracts by conventional electrophoretic methods combined with infectivity titrations.

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[2] M. K. BRAKKE, J. Amer. chem. Soc. *73*, 1847 (1951).

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Electrophoretic methods in virus research. Smaller viruses consist of nucleic acid with a protein coat. Some viruses also contain lipid, but in all probability their surface is mainly, if not entirely, made up of protein. Consequently, the advantages of electrophoresis in the study of proteins should persist for viruses as well.

Compared to other branches of protein chemistry, virology has hitherto gained remarkably little from applications of electrophoretic methods. This is true for purification as well as for characterization of viruses. It seems that this can be partly explained by the fact that electrophoretic methods are incapable of yielding any molecular data, while electrochemical data have not aroused the same interest. Furthermore, hitherto used electrophoretic methods are not ideally suited to work with viruses.

Potentialities of electrophoretic methods in general. Like centrifugation, electrophoresis is an extremely mild method for purification, much to be preferred to precipitating agents, organic solvents etc. Its independence of molecular size and shape makes it a wonderful complement to differential sedimentation, because a preparation with well-defined ultracentrifugal characteristics may well display a whole spectrum of components with different charge characteristics. Thus a preparation of tobacco mosaic virus that appeared homogeneous in the ultracentrifuge was shown by SINGER et al.[6] to represent distinct molecular species when analyzed by electrophoresis.

The electrophoretic method of characterization is very sensitive to small differences or changes in a protein molecule, provided that they affect the net charge of the particle within its stability pH range. Thus hemoglobin S (from sickle cells) can be distinguished from normal hemoglobin electrophoretically at pH 7, although it is known that these two molecular species differ by one single amino acid in a subunit of the molecule, resulting in a difference of 2 charges per molecule. This corresponds, for hemoglobin, to a shift in the isoelectric point of 0.2 pH units. The precision of a measurement of isoelectric point is better than that, about 0.1 pH unit.

In the virus field, mutants can sometimes be differentiated by electrophoretic means. Thus MAC DONALD et al.[7] showed that a spontaneous mutant of southern bean mosaic virus had a pH-mobility curve different from that of the parent strain. GINOZA and ATKINSON[8] investigated the isoelectric points and pH-mobility curves of eight strains of tobacco mosaic virus and could thereby confirm an earlier classification into four groups. A systematic electrophoretic study of viruses might furnish new criteria for their classification.

Possible differences in the electrophoretic mobility of biologically closely related viruses, such as the oncogenic viruses of mice or chickens, might prove to be of great value. In experimental medicine, it is sometimes highly desirable to separate viruses in tissue culture fluid or tumor extracts. For example, on injection of extracts of leukemic tissues of mice and chickens, non-leukemic tumours have sometimes been obtained as well as leukemias. The presence of two distinct viruses has been proved in mice (GROSS[9]; STEWART[10]), but so far not in chickens. Electrophoresis or chromatography might be able to establish whether in chickens one virus causes the two different tumours, or if different viruses are involved.

Another important application of electrophoresis consists in the correlation of various biological properties of a virus, such as infectivity, interference, hemagglutination, complement fixation, and immunoprecipitation, with one or more electrophoretic particle populations. Many other possible applications are concerned with changes occurring when viruses are treated with formaldehyde (FISCHER and LAUFFER[11]), alkali (SCHRAMM et al.[12]), and other chemicals.

Microscopic electrophoresis[13] is based on direct microscopic observation of migrating carrier particles of quartz or collodion on which the protein to be studied has been adsorbed. Such particles acquire the same mobility as the coating protein has in solution. However, the material to be measured has to be very pure, otherwise the carrier particles may be partly or preferentially coated by the impurities. If this condition is satisfied, the method lends itself excellently to determination of isoelectric points and pH-mobility curves of viruses, since the accuracy of these measurements is satisfactory and the amounts of pure virus required are extremely small. Another important advantage of the method is the possibility of measuring mobilities of microscopically visible aggregates of viruses insoluble around the isoelectric point; sorption on carrier particles is then superfluous. Applications of this technique have been presented by OSTER[14], MILLER et al.[15], and SMADEL et al.[16]. A recent review of the method and its applications has been given by BRINTON and LAUFFER[17]. It is not suitable for preparative purposes.

[6] S. J. SINGER, J. C. BALD, S. G. WILDMAN, and R. D. OWEN, Science *114*, 463 (1951).
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[15] G. L. MILLER and W. C. PRICE, Arch. Biochem. *11*, 337 (1946).
[16] J. E. SMADEL, E. G. PICKELS, and T. SHEDLOVSKY, J. exp. Med. *72*, 523 (1940).
[17] C. C. BRINTON, JR., and M. A. LAUFFER, *Electrophoresis, Theory, Methods, and Applications* (Ed. M. BIER, Academic Press, New York 1959), p. 427.

Moving boundary electrophoresis is well suited to electrochemical characterization of pure or almost pure virus preparations. With the best instruments available, mobility values accurate to 10^{-6} cm²/volt sec and better are easily obtained, and, as already mentioned, isoelectric points can be determined to less than 0.1 pH unit provided the virus is sufficiently soluble and can be identified with certainty with an optically visible component.

Studies of this kind have been performed by many investigators. However, many of them have only measured the mobility at a single pH and salt concentration, which is useful for identification purposes but very incomplete as a characterization. BRINTON and LAUFFER[17] have presented a list of hitherto known isoelectric points of viruses; they all lie in the range between pH 3.5 and 6.

The moving boundary method requires comparatively much purified virus material. In the big commercial instruments, about 20 mg is needed for a disturbance-free electrophoretic run, but the material can be saved and re-used in several runs or for other purposes. Nevertheless, this quantity of pure virus is in many cases prohibitively large. In table-size instruments, the quantity required is less, being of the order of 2–5 mg.

Attempts to carry out electrophoresis experiments by Tiselius' method at very low concentrations of pure virus can lead to serious difficulties, and one has to be careful not to lose the costly material (SHEDLOVSKY and SMADEL[18]). The electric current tends to develop irregular thermal convection and laminar electro-osmotic streamings in the electrophoresis channel. These effects actually take place whereever no hydrodynamically stabilizing effect is active. In moving boundary electrophoresis, the only stabilizing effect available is the density gradient accompanying the moving boundaries. Consequently, at a given electric load, there is a minimum concentration of the migrant that can be used. For elementary bodies of vaccinia, SHEDLOVSKY and SMADEL found this critical concentration to be as high as 0.2% by weight, the limiting factor being electro-osmotic streamings, not thermal convection. In the presence of protein with about the same mobility as the elementary bodies, the disturbance was found to disappear. The weak virus boundary was then protected by the additional density gradient given by the protein.

Several authors have reported comparative electrophoretic studies of extracts from diseased and healthy plants or organs (FRAMPTON and TAKAHASHI[1]; TAYLOR et al.[19]). Even if distinct and reproducible differences are found, which possibly can be used for diagnosis, it is always risky to draw conclusions regarding the electrophoretic behaviour of the virus itself from such investigations. A sharp (slowly diffusing) peak in the diagram of the pathologic extract may or may not be the virus. It may also be one of several virus components. No conclusion is safe without infectivity assays on electrophoretic fractions. In preparations not extensively purified, the virus concentration is far below the resolution limit of the best optical methods. Infectivity assays are then necessary to get mobility values.

Such measurements on viruses in the Tiselius apparatus have been described by BOURDILLON and LENNETTE[20], BOURDILLON[21], HARTMAN and LAUFFER[22], and RANDRUP[23]. The crucial difficulty rests in the technique for taking fractions for biological tests. Special sampling devices for the Tiselius apparatus have been described by BOURDILLON and LENNETTE[20], HARTMANN and LAUFFER[22], and RANDRUP[24]. In the latter paper, the sampling accuracy is investigated in detail, and it is shown that the mobility of a virus can be measured to within 10% despite the fact that the relative accuracy of the titration methods is much lower. By keeping the virus boundary stationary in the U-tube by a controlled back-flow of buffer, the electrophoretic run can be much prolonged; the accuracy in the mobility measurement then increases accordingly.

Some difficulties in the use of a Tiselius apparatus for virus analysis by sampling and infectivity titration will be discussed here. The oblong form of the cuvettes, so advantageous for optical analysis, is not very suitable for taking samples through a capillary. The liquid close to the front and rear walls has to flow horizontally a distance of 12 mm before it reaches the capillary tip. Thus an appreciable density gradient is necessary for a successful sampling, and the purer the virus, the less accurate becomes the sampling. The strong density gradients, used by RANDRUP[24] in his assessment of the sampling accuracy, are in general not available in actual experiments, at least not when purified preparations are concerned.

Optical analysis is only of secondary interest in virus analysis by infectivity titration of fractions. To improve the flow characteristics round a sampling capillary, therefore, it would be preferable to use a U-tube with a cylindrical cross-section. This was the usual procedure before the break-through of Tiselius' method (see for instance TODD[25]) and was suggested more recently by POLSON[26], yet without much consequence for virus electrophoresis methodology.

Although RANDRUP's technique of removing the sampling needle for each fraction seems cumbersome

[18] T. SHEDLOVSKY and J. E. SMADEL, J. exp. Med. *72*, 511 (1940).
[19] A. R. TAYLOR, D. G. SHARP, D. BEARD, and J. W. BEARD, Proc. Soc. exp. Biol. Med., N. Y. *51*, 137 (1942).
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[26] A. POLSON, Nature *145*, 27 (1940).

and liable to cause local convection, it is probably about the best that can be done in a standard Tiselius apparatus. Modified U-tubes with fixed capillary sampling tubes at the side of the limbs have been described by SVENSSON[27,28] and KEKWICK et al.[29], but have not been applied to viruses. Undoubtedly, such equipment would allow a much more complete mapping of virus activity versus electric mobility than a standard U-tube. However, the possibilities of detecting two or more virus components by the moving boundary method are nevertheless rather restricted, for the following reasons.

In the term 'moving boundary electrophoresis' lies the fact that the result of the migration is a separation of front and rear *boundaries* only, while the *zones* containing the various constituents still overlap (Fig. 1). Let us now suppose that a certain virus preparation contains two infective components of different mobilities, and that their relative concentrations, in infectivity units, are such that the fastest component can be regarded as the main, the slowest one as a minor constituent. Going from top to bottom in the ascending limb, then, the infectivity will be expected to rise from zero to a high level at the fastest virus boundary, and then to rise a little more at the second boundary. However, *the second, small rise cannot be detected experimentally* because of the rather low relative accuracy of virus titration methods. The standard deviation of such measurements is actually so great that it is difficult to ascertain changes in infectivity smaller than $\pm$ 50%.

The two components can, however, be detected in the other limb, because there the boundary of the minor component comes first. The ultra-high sensitivity of the biological assays secures the detection of the minor constituent, while the rise in infectivity at the boundary of the main component is, according to our assumptions, well over 50%.

To sum up, moving boundary electrophoresis in conjunction with infectivity titration of fractions is capable of demonstrating the presence of two infective electrophoretic fractions in one limb, provided that the number of fractions titrated is great enough, but never in both limbs. If the components are of about the same titer, there is a risk that the second one remains undetected in both limbs. Only the levels at which infectivity first appears may then give an indication of the presence of more than one infective component. In the case of three such components, similar considerations lead to the conclusion that one or two components will escape detection in at least one limb of the U-tube.

As far as known to the authors, nobody has ever titrated a sufficient number of fractions from a moving boundary experiment for proving the electrophoretic homogeneity of a virus preparation.

A certain purification, as well as separation of virus components, can be achieved by the moving boundary method. However, since the material in the bend of the U-tube (Fig. 1) always retains its original composition, the method is not very effective.

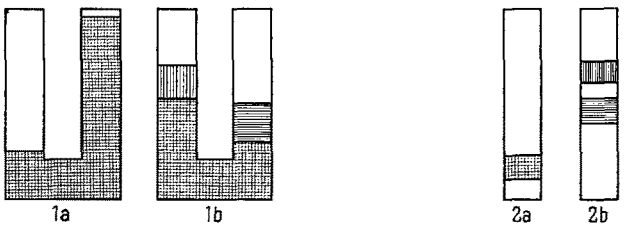

Fig. 1 and 2. Separation of two virus components in moving boundary (1) and in zone (2) electrophoresis.
a) Distribution before electro-phoresis; b) Distribution after electrophoresis. Virus component indicated by dots has a higher mobility than component indicated by lines.

Zonal electrophoresis methods. The limitations of the moving boundary method discussed above have caused difficulties not only in virus research, but in other applications as well. During the last decade, therefore, there has been an extensive development of various methods for *zonal* electrophoretic separation. The principle, illustrated in Figure 2, consists in having a conducting channel stabilized against convection, in which a narrow zone of the sample is introduced. Under the influence of the current, the various components are split up into discrete zones, completely separated if a sufficient time is allowed.

Considering again the case of two electrophoretic virus components, it is easily realized that both can be detected and localized, whatever their relative concentrations are. Provided that the constituents are well separated and that a sufficient number of fractions are titrated, the bad relative accuracy of virus titrations does not come into play, while use is made of the high sensitivity. Zonal electrophoresis is thus ideally suited to virus analysis.

According to the method used for hydrodynamic stabilization, these methods can be subdivided into paper electrophoresis, gel electrophoresis, electrophoresis in powder (starch, cellulose) columns or slabs, density gradient electrophoresis, liquid film electrophoresis, laminar flow electrophoresis, and rotating tube electrophoresis. The three last versions have been very little developed and will not be discussed here. Some view-points regarding the application of the others to virology will be given below.

[27] H. SVENSSON, Ark. Kemi Min. Geol. *22A*, No. 10 (1946).
[28] H. SVENSSON, Ark. Kemi Min. Geol. *15B*, No. 19 (1942).
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Paper electrophoresis has grown to an immense importance in protein chemistry during the last five years. In view of the copious literature in this field, it is very remarkable that almost nothing has been published on paper electrophoresis of viruses. Probably many investigators have tried, but met with difficulties or got inconclusive results which have not been published. Extensive sorption to the paper fibers is the probable cause of such difficulties. Sorption gives rise to tailing of migrating zones and to difficulties in obtaining complete elution of virus from the paper.

That sorption of virus to paper is a reality and not only a hypothesis is shown very nicely in a paper by ZAITLIN[30], although his application of paper electrophoresis was completely successful. He used continuous electrophoresis in a vertical paper curtain and demonstrated that two strains of tobacco mosaic virus could be completely separated. However, the relative amounts of the two strains collected from the curtain did not correspond to those fed to the starting point. On the other hand, when the experiment was interrupted and the curtain dried and stained for protein, the relative amounts of bound dyestuff was wrong in the other direction. This proves that one of the strains was sorbed to the paper much more strongly than the other. In the continuous technique, a reversible sorption effect does not impair the separation and purification, because the sorption influences the vertical flow as much as the horizontal migration. Sorbed or free, all molecules then stick to the same migration path through the curtain. Quantitative analysis, however, is only possible after complete elution of the virus from the paper by allowing buffer and current to flow for a considerable time after the flow of virus suspension has been stopped.

Electrophoresis in gels and powders can be expected to offer the same difficulties when applied to viruses. Even here the information available in the literature is very scanty. HIRTZ and CAMAND[31] have investigated the electrophoretic behaviour of foot-and-mouth disease virus in a starch trough, the migration being analyzed by measurement of complement fixation. They found at least two complement-fixing components in each strain, and no complications due to sorption are apparent from their report.

Density gradient electrophoresis. In this technique, hydrodynamic stability is obtained in a vertical column of free liquid by preparing the latter in such a way that the density steadily increases from top to bottom. Thus the column is characterized by a density gradient, which effectively opposes the current's tendency to develop thermal convection and electroosmotic streaming. The increasing density is generally obtained by arranging for an increasing concentration of sucrose.

Sorption and molecular-sieving effects are completely absent in this method. In contrast to the moving boundary method, there is no lower limit to the concentration of the sample to be analyzed. As a matter of fact, the method works better and safer the lower the concentration is.

Since the density gradient stabilizes the liquid even after the run, no stop-cocks or other mechanical means for subdividing the column into compartments are necessary. The contents can simply be emptied slowly through a stationary capillary, from the other end of which any number of fractions can be taken. The curve relating virus activity with the level in the column can thus be obtained in corresponding detail. Because of the complete separation into discrete zones, enough material can be obtained for testing different biological properties of the virus in each fraction. The electrophoretic resolution of components is good. The removal of the virus from the column is practically quantitative, the only possible losses being due to a slight adsorption to the walls of the column and the sampling capillary.

Mobility values can only be obtained by inclusion of (preferably coloured) reference substances with known mobilities. This limitation the method shares with those using solid support. Hemoglobin and bromophenol-blue-stained serum albumin can be recommended as reference substances. Since serum albumin is the only component of serum that combines with bromo-phenol-blue in the native state, the practical procedure is to add hemolyzed serum and a very small quantity of bromo-phenol-blue to the virus sample. The addition of two such reference substances allows the construction of a mobility spectrum of virus activity with a fair accuracy.

The experimental facts and the considerations based thereon which have been reviewed on the preceding pages reveal without doubt that electrophoresis in general is capable of rendering much more service to virology than has been realized hitherto. This statement refers to purification as well as to electrochemical characterization of viruses. Moreover, it is evident that, at the present state of development of electrophoretic methods, the density gradient technique offers quite special advantages to virus research that cannot be achieved by any other electrophoretic procedure in current use. For this reason, a survey of the results gained hitherto, during a couple of years only, in virus research by density gradient electrophoresis will be presented in the next section.

Experiment techniques for density gradient electrophoresis. Various methods and apparatus for density gradient electrophoresis have been reviewed recently by SVENSSON[32]. A short description of the technique

[30] M. ZAITLIN, Biochim. biophys. Acta *20*, 556 (1956).
[31] J. HIRTZ and R. CAMAND, Rev. Immunol. *18*, 206 (1954).
[32] H. SVENSSON, *A Laboratory Manual of Analytical Methods in Protein Chemistry, including Polypeptides* (Eds. P. ALEXANDER and R. J. BLOCK, Pergamon Press Ltd., London 1960), Vol. 1, p. 193.

used for work with viruses by CRAMER et al.[33–36] is given below.

The apparatus used in these investigations is shown in Figure 3. An U-tube provided with a wide stop-cock in one limb is connected to two electrode vessels with capillaries at their lower ends. One limb of the U-tube has a vertical extension which is closed by a rubber stopper through which passes a capillary sampling tube A and a drainage tube B. Sometimes two sampling capillaries were used. The electrodes are silver wires coated by silver chloride and are always surrounded by a concentrated chloride solution.

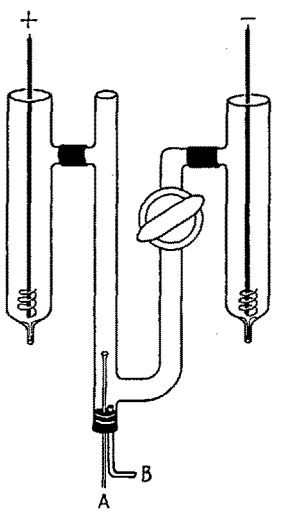

Fig. 3. Electrophoresis apparatus

The apparatus is first filled with buffer solution to a level slightly above the tubes connecting the U-tube with the electrode vessels. A solution of the same buffer composition and containing in addition 40% sucrose is then introduced through tube B and is allowed to rise to the level of the tip of tube A. The stop-cock is then closed, and the positions of the menisci are marked outside the electrode vessels.

In the gradient-mixing device shown in Figure 4, the flask P_1 is filled with buffer without sucrose, and P_2 with heavy buffer containing 40 % sugar. The device is placed at a level higher than the menisci in the apparatus, and the outlet tube A is connected to the capillary tube A in Figure 3. After a magnetic stirrer in flask P_1 has been started, the contents of the mixer are allowed to run into the apparatus slowly. The mixer delivers a solution with a steadily increasing sucrose concentration, which displaces the light buffer in the apparatus towards the left-hand electrode vessel. The level of the meniscus is readjusted to the mark.

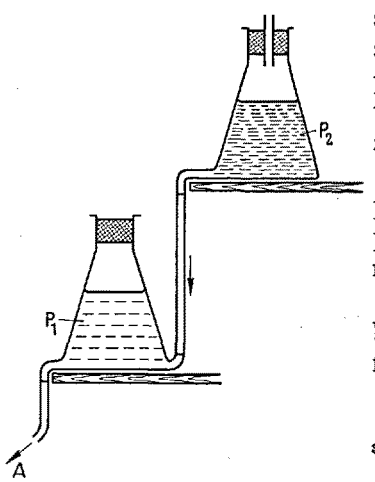

Fig. 4. Gradient mixing device made of two Erlenmeyer flasks, P_1 containing buffer, and P_2 containing 40% sucrose in the same buffer.

Saturated sodium chloride solution is next pressed into the electrode vessels until the electrodes become completely covered by it. After the menisci have again been readjusted to the marks, the stop-cock is cautiously opened. Because of the increased hydrostatic pressure in the left-hand limb, the menisci will now assume a small level difference, and the original boundary between heavy and light buffer will be shifted somewhat downwards.

The apparatus is now ready for receiving the virus sample, but before that one has to be sure that the latter has a suitable density. To that end, two Vidal tubes are prepared, one containing the heavy buffer solution with 40% sucrose, the other a sample from the column obtained by temporarily opening tube A in Figure 3. Sucrose is dissolved in the virus suspension to give approximately 35%. The sample has a suitable density if one droplet thereof floats on the surface in the first Vidal tube and sinks to the bottom in the other. The sucrose concentration has to be adjusted up or down until this condition is satisfied. The virus sample can then be pressed into the column through the capillary tube A.

After about 20 min, diffusion of sucrose across the initial virus zone has created a density gradient within it strong enough for starting the current. The duration of the electrophoretic run depends on the virus material. Short runs of only 4 h have been performed with very unstable viruses, whereas in general the duration has been extended to about 16 h, with a current of 15 mA and a potential gradient of 3.5 V/cm. A cooling system adapted to the electrophoresis apparatus has been constructed, allowing work at a constant temperature of 4° C and less. Substances stabilizing the biological activities of viruses can be added to the whole column. Rabbit hemoglobin and phenol red, when added to the virus suspension in some experiments, were useful guides to the progress of the migration.

After electrophoresis the stop-cock is closed, and the column is emptied through capillary tube A and subdivided into fractions. Since traces of virus may stick to the wall of this capillary from the injection procedure before the run, it may be advisable to use two capillary tubes, one for injection of the initial zone, the other for sampling.

In order to describe more fully the experimental procedure, a specific example of an experiment with poliomyelitis virus will also be related. This virus is relatively thermoresistent.

Poliomyelitis tissue culture fluid was concentrated by pervaporation (POLSON and HAMPTON[37]) and purified by three cycles of differential ultracentrifugation.

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[34] A. POLSON and R. CRAMER, Biochim. biophys. Acta *29*, 187 (1958).
[35] R. CRAMER, Nature *183*, 195 (1959).
[36] R. CRAMER and S. STEWART, Proc. Soc. exp. Biol. Med., N. Y. *103*, 697 (1960).
[37] A. POLSON and J. W. F. HAMPTON, J. Hyg., Camb. *55*, 344 (1957).

To 0.3 ml of this concentrate with a titer of $10^{7.85}$ ID 50 per 0.1 ml, 0.7 ml of a 35% sucrose solution in buffer and one drop of rabbit hemoglobin was added. The material was introduced carefully by a syringe through the capillary A, Figure 3. Due to the red colour, the outflow of the virus-containing material above the tip of the capillary was easily controlled. After the virus sample, heavy buffer solution was injected through the same capillary till the virus material had left the capillary and formed a flat layer 1–2 mm thick between the 40% sugar level below and the sugar gradient above. 20 min later electrophoresis was started and allowed to proceed for 16 h at room temperature. Then the stop-cock was closed and the column fractionated into samples of 0.5 ml each, using a clean capillary as recommended above. The virus activities of the samples were determined by injection of 10-fold dilutions into groups of 5 tubes of trypsinized monkey kidney cells. Each tube received 0.1 ml. The 50% infectivity end-points were calculated by the method of REED and MUENCH[38]. The infectivity titers found for each sample are indicated in Figure 5. The amount injected into the column was 8.32 log TCID 50, while that recovered was 8.09 log TCID 50. The recovery of virus activity was thus about 58% in this experiment.

Review of results obtained by the density gradient method. BRAKKE[39] was the first to use density gradient electrophoresis for viruses. CRAMER, LERNER, and POLSON[33] applied the method to the study of the FA strain of mouse encephalomyelitis virus. So far the Stockholm and Cincinnati strains of poliomyelitis virus type I (POLSON and CRAMER[34]), the Rous sarcoma virus (CRAMER[35]), and a hemagglutinating as well as a masked strain of polyoma virus (CRAMER and STEWART[36]) have been examined by the same method. Some results will be discussed here in order to demonstrate the value of the method for work with viruses. The main purpose of these studies was to gain possible new criteria for their *classification by determining their electrophoretic mobilities.* For example, Theiler's virus, which is very closely related to the group of poliomyelitis viruses, exhibits an entirely different mobility. POLSON (personal communication) has succeeded in an electrophoretic classification of the ECHO, poliomyelitis, and Coxsackie virus groups. A similar study of the cancer viruses has been started by one of us (CRAMER). The viruses are being characterized by their relative mobilities, that is, their migration distances compared with those of added reference substances, such as rabbit hemoglobin or neutral red; their mobilities have been accurately measured in the Tiselius apparatus.

A phenomenon that has to be discussed in some detail is the *electrophoretic inhomogeneity* found for some viruses, e.g. poliomyelitis (Fig. 5) and Rous sarcoma virus (Fig. 6). Such results were already suspected on the basis of inhomogeneity found earlier for some viruses when analyzed by other methods, such as immunoprecipitation (LE BOUVIER, SCHWERDT, and SCHAFFER[40]; POLSON, EHRENBERG, and CRAMER[41]) or analytical centrifugation (POLSON, EHRENBERG, and CRAMER[42]). The possibility that the electrophoretic inhomogeneity might be due to an artefact was very carefully considered. On the one hand, an artefact due to the sampling technique was excluded by the following experiment. Poliomyelitis virus was introduced into the electrophoretic column and displaced to a position about 6 cm above the capillary tip in Figure 3.

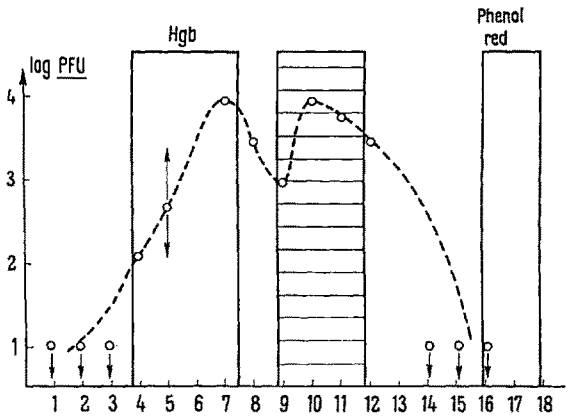

Fig. 5. Zone electrophoresis diagram showing the distribution of Rous sarcoma virus activity in log plaque-forming units (PFU) for each consecutive electrophoretic fraction of 0.8 ml each, indicated on the abscissae. The positions of the reference substances are indicated by the first and third vertical columns and the opalescent zone containing the impurities by the second vertical column. [Reproduction from CRAMER, Nature *183*, 195 (1959).]

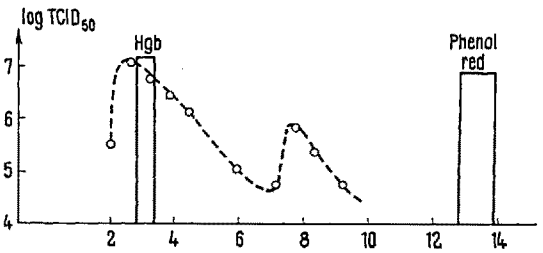

Fig. 6. Electrophoresis diagram of purified Stockholm type I poliomyelitis virus. The vertical columns indicate the positions of the reference substances. The virus titers are given in log TCID-50 units on the ordinate for each consecutive fraction of 0.5 ml, fraction numbers being along the abscissae. [Reproduced from POLSON and CRAMER, Biochim. biophys. Acta *29*, 189 (1958), with the permission of the journal.]

[38] L. J. REED and H. MUENCH, Amer. J. Hyg. *27*, 493 (1938).
[39] M. K. BRAKKE, J. Amer. chem. Soc. *73*, 1847 (1951); Arch. Biochem. Biophys. *45*, 275 (1953).
[40] G. L. LE BOUVIER, C. E. SCHWERDT, and F. L. SCHAFFER, Virology *4*, 590 (1957).
[41] A. POLSON, A. EHRENBERG, and R. CRAMER, Biochim. biophys. Acta *29*, 622 (1958).
[42] A. POLSON, A. EHRENBERG, and R. CRAMER, Biochim. biophys. Acta *29*, 612 (1958).

When the virus was sampled from this position, only one peak of virus activity was found. On the other hand, the development of an artefact during electrophoresis due to convection currents before sampling does not seem to occur, for two reasons. First, when the fast-moving fraction of poliomyelitis virus was separated from the slow one and subjected to a repeated run, it exhibited the same mobility, and no slow activity peak was found. Second, certain viruses were found consistently to be electrophoretically homogeneous, that is, to have a single mobility, which again excludes an artefact as the factor underlying the electrophoretic inhomogeneity found for other viruses. It seems, therefore, that the assumption that highly purified virus populations must always be homogeneous when tested by various physical methods should be reconsidered. The finding of inhomogeneous populations is attributable to the application of improved analytical methods.

Correlation of the *electrophoretic mobility* of a virus with its *degree or mode of purification* hast just begun. Identical electrophoretic diagrams were recorded for native and highly purified poliomyelitis virus type I. For this virus, therefore, no measurable changes in mobility due to removal of impurities or due to changes attributable to the purification procedure seem to occur. On the other hand, slightly different diagrams were obtained for Theiler's virus when different purification methods, such as the ether and chloroform methods, were used. No other comparative data on viruses purified by different methods are known to the present authors. However, it might be of value to study such effects systematically.

It is not yet clear how the finding of apparently distinct electrophoretic populations for a virus of biologically well-defined specificity, as has been found for poliomyelitis virus type I (POLSON and CRAMER[34]) and Rous' sarcoma virus (CRAMER[35]), is to be interpreted. The biological specificity of the former virus is demonstrated by the fact that, for both the Stockholm and the Cincinnati strain, the tissue culture supernatant used for the electrophoretic experiments exhibited a cytopathogenic effect in tissue culture specifically neutralized by type I poliomyelitis antiserum. Likewise, the tissue culture supernatant used for electrophoresis of Rous' sarcoma virus induced pocks on the chorioallantoic membrane, which is highly specific for Rous' virus, but did not induce these tumours when previously incubated with anti-Rous-sarcoma serum from a chicken with regressing Rous tumour.

The possibility that electrophoretically distinguishable virus fractions may exhibit some difference in biological specificity cannot be excluded.

POLSON and CRAMER[34] found that the relative abundance of the electrophoretically faster and slower components of poliomyelitis virus type I was significantly different between the avirulent Cincinnati and the highly virulent Stockholm strain. This discovery is interesting, but continued investigations including *in vivo* experiments are required before any definite conclusions can be drawn.

Biological properties, probably related to the surface of a virus particle, have been examined, but so far only to a very limited extent. The binding of homologous antibody to the surface of the virus is such a property. Preliminary results (unpublished) for poliomyelitis virus type I do not show any difference in neutralizability between the two electrophoretically distinguishable virus populations. Another immunological method, immunoprecipitation, was used in the case of Rous' sarcoma virus (unpublished). Each electrophoretic fraction was tested in agar by the method of OAKLEY and FULTHORPE[43]. Serum from chickens with regressing Rous tumours and with known neutralizing capacity for Rous' virus was used. As indicated in Figure 7, very faint precipitation lines were recorded, usually only in fractions containing much infective virus. In order to test if the antigens of the two electrophoretic populations were identical

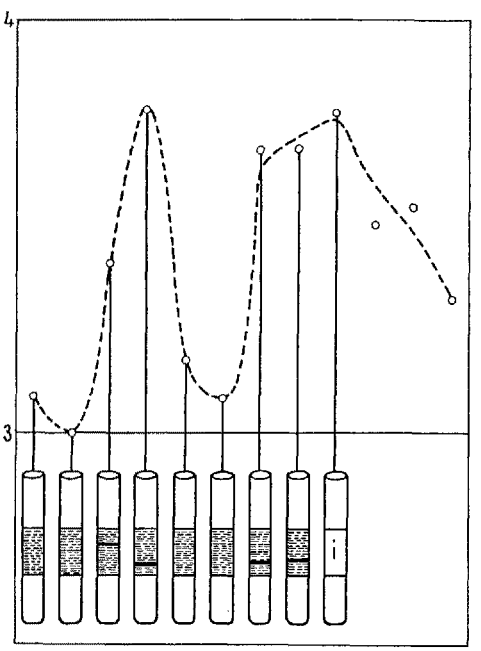

Fig. 7. Zone electrophoresis diagram showing the distribution of Rous sarcoma virus activity in log plaque-forming units for each consecutive electrophoretic fraction as indicated on the abscissae. The tubes show results of immuno-precipitation tests performed for each fraction, according to OAKLEY and FULTHORPE. The middle part of the tube represents a neutral zone of 0.9% agar, resting on a zone containing 0.9% agar mixed with serum of a chicken with regressing Rous tumour and previously determined neutralizing capacity for Rous virus. A sample of each electrophoretic fraction had been layered above the neutral zone. Faint precipitation lines appeared after two weeks with the fractions of highest virus activity.

[43] C. L. OAKLEY and A. J. FULTHORPE, Arch. Biochem. *11*, 337 (1953).

or not, a confrontation of the corresponding antigens by the Ouchterlony technique was attempted, but so far no precipitation lines have been obtained in this system.

A combined electrophoretic and immunologic analysis is already available in the form of the ingenious immunoelectrophoretic method described by GRABAR and WILLIAMS[44]. The applicability of this method to viruses has not been demonstrated; probably sorption and molecular-sieving effects will cause difficulties. In density gradient electrophoresis, such difficulties are entirely out of question. Consequently, it may be more advantageous for virologists to try a combination of density gradient electrophoresis and the immunoprecipitation technique described by OUCHTERLONY[45] and OUDIN[46].

Density gradient electrophoresis has proved to be of value also for the *purification of viruses*. For poliomyelitis virus, it has been found that the active agent separates well from a fastermoving opalescent zone. For Rous' virus, a first peak of virus activity was found to separate from a faster migrating opalescent zone still containing some virus activity. A separation of the active agent from two opalescent zones has been observed for polyoma virus as well. The study of many viruses, especially cancer viruses, is still seriously hampered by the lack of satisfactorily purified virus preparations. The purification effects obtained for unstable viruses, such as Rous' sarcoma virus, which are not resistant to organic solvents, are of special interest.

It is sometimes desirable to remove not only impurities, but also biologically active material, such as *virus inhibitors*, from a virus preparation. In the case of a masked non-hemagglutinating strain of SE polyoma virus, a definite separation of the hemagglutinin from an inhibitor substance has been achieved (CRAMER and STEWART[36]).

From the above considerations, it is evident that electrophoretic methods are capable of rendering a greatly increased service to virus research. Although this statement refers to all electrophoretic methods in current use, the authors are convinced that the density gradient method offers special advantages to workers in the virological field. The viruses, being excessively large molecular entities, are more susceptible to sorption and molecularsieving disturbances than are smaller molecules of biological interest, when electrophoresis in paper, powders, or gels is tried. The density gradient method rules out these secondary influences altogether. Moreover, a maximum of sampling accuracy is ascertained by this method, and no problems concerning quantitative elution of active material are relevant. The fractions obtainable from density gradient runs can be assayed by a multitude of biological tests, which is very essential for work with viruses.

Résumé. Les avantages de la méthode d'électrophorèse en gradient de densité pour des travaux en virologie sont analysés.

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[45] O. OUCHTERLONY, Acta path. microbiol. scand. *25*, 186 (1948).
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Renal and Extrarenal Actions of Aldosterone*

By F. GROSS**

The isolation, identification, and the synthesis of aldosterone were accomplished in a situation and under conditions quite different from those existing for other adrenocortical hormones. Already before the genuine hormone became known, there was a steroid – cortexone – isolated from the adrenal cortex, the action of which corresponded quite well to the concept of the rôle which the natural hormone would play in sodium metabolism. Some investigators even went so far as to claim cortexone to be the essential secretory product of the adrenal cortex. Hence, when the natural hormone was recognised, it met a biased situation in so far as it was expected to produce the same effects as its predecessor which was already designated as 'sodium retaining' or, even worse, as 'mineralocorticoid'. The fact that, due to the complicated synthesis, only very small quantities of aldosterone were available for several years had the consequence that the majority of investigations of this hormone concern its excretion in the urine and to a lesser degree its secretion rate in various normal and pathological conditions. It is understandable that in view of the characterisation as a sodium retaining hormone, most efforts in this research were directed towards the detection of an elevated aldosterone secretion in pathological conditions associated with a retention of sodium or with edema formation. The syndrome of primary aldosteronism was

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