Electrophoresis using a constant potential across the separation field

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Reproducibility of electrophoretic runs depends on the maintenance of a smooth and constant voltage gradient within the supporting medium or separation chamber. Power packs to supply constant current or constant voltage as measured at the pack are readily available, but if such a pack is used with constant voltage, the proportion of the voltage drop available for the electrophoretic separation will be affected not only by changes in the resistance in the separation field, but also by changes in the external circuit, which will be certain to occur at the electrodes, separation membranes, or buffer reservoirs. For this reason, it is usual to work with constant current, since the voltage drop in the separation field will then be independent of the resistance changes in the external circuit.

Brattsten (1955) demonstrated that the effect of temperature variation on migration velocity may be greater where constant voltage is used and recommended constant current. He was using a system without a supporting medium. Where supporting media are used there are many variables in addition to temperature. Under constant current conditions any change affecting the resistance within the supporting medium or separation chamber will cause a variation in the voltage gradient and consequently a lack of reproducibility from one run to another. In continuous electrophoresis, either with or without a supporting medium (as in the Elphor VaP apparatus), such variation leads to a reduction of the efficiency of separation. In micro-immuno-electrophoresis in agar the thickness of the agar and the buffer retention might vary from one batch of slides to the next. Supporting media, such as cellulose acetate, cellogel, or paper which have to be 'blotted' to remove surplus buffer, do not provide exactly reproducible conditions; ionic pile up at membranes or edges of supporting media will also affect reproducibility, and the heat produced during a run may cause progressive changes in supporting media. All these effects are even more pronounced with zone electrophoresis in agar, starch grain, or pevikon block.

A control method providing constant potential over the separation field is described in the present paper and shown to be capable of giving reproducible results in many kinds of electrophoresis, even under conditions where fluctuations of mains voltage have been so severe that conventional power packs have been quite useless.

SUPPORTING MEDIA AND APPARATUS

1 CELLULOSE ACETATE (Oxoid Ltd., London) Strips 20×5 cm. or 10×2.5 cm., were used in the Kohn Mk. 1 electrophoresis bath (Shandon, London). The technique was as described by Kohn (1961), except for the current densities and separation times. The runs were carried out at room temperature.

A 1 cm. wide strip of Whatman no. 1 or 3 MM filter paper was placed across the bath in place of the strip no. 1 (Kohn, 1964, personal communication). When using constant potential over the separation field control the sensing electrodes were touching either end of this strip.

2 CELLOGEL (Chemetron, Milan) This is a gelatinized form of cellulose acetate which was preferred for the separation of certain globulins (Bidwell, Dike, and Denson, 1966).

3 AGAR IMMUNO-ELECTROPHORESIS This was carried out on 7.5×2.5 cm. microscope slides following the method of Augustin and Hayward (1960) using 1% Oxoid agar or Oxoid Lonagar containing barbiturate buffer at final $\mu = 0.025$. The Shandon Kohn Mk. 1 tank was used with a sheet of Perspex (I.C.I.) across the compartment divisions to support the 8 or 16 slides on each run. When constant potential over the separation field control was used a dummy Perspex slide either 3 in. or 6 in. long was placed among the other slides, there being platinum contacts at either end of this slide for potential sensing.

4 STARCH GRAIN (Bloemendal, 1963) Blocks, $25 \times 16.5 \times 1.5$ cm., were made in a Perspex tray with two thicknesses of Whatman seed test card (Reeve Angel Ltd., London) embedded in either end as wicks. The sides of the tray were removed during the run, and the supporting medium was covered with a single thickness of 0.025 mm. polyethylene. The substance to be separated was made up in a thick slurry of the supporting medium and placed in a 1 cm. deep and wide slot cut in the block 5 cm. from the cathode end of the block. The block was placed horizontally in a large tank that held a total of 8l. of buffer between the four compartments. Coiled platinum electrodes were used, and short pieces of platinum wire were inserted into the ends of the block for potential sensing. The runs were done at $2-4^\circ \text{C}$.

In experiments with serum or plasma the albumin was stained with bromophenol blue to permit visual assessment of the shape of the advancing front and, at completion of the run, the vertical form of the band.

On the completion of the run the block was cut into 1 or 2 cm. sections parallel with the origin, eluted with a suitable buffer and concentrated by ultrafiltration. Cellogel electrophoresis was carried out on the concentrates to identify the constituents.

5 PEVIKON (Bocci, 1962) The pevikon was washed three times with acetic acid and then with many changes of water to remove as much of the plasticiser as possible, although foaming was not prevented by the most

exhaustive washing. The method used for starch grain was followed.

6 THE ELPHOR VAP HIGH VOLTAGE FREE BUFFER FILM APPARATUS This apparatus was developed from that described by Hannig (1961), and was supplied by Bender/Hobein, Munich.

Ion exchange membranes were used between the separating chamber and the electrode compartments. The potential gradient applied when using constant potential over the separation field was 46 V/cm. With the constant current power pack the initial experiments were carried out at 150 mA. Sensing electrodes were introduced through buffer inlet points 1 and 6 and sample introduction point 2 (the physical centre of the width of the plates) to monitor and later to control the voltage across the plates. The electrode at the centre point was connected, via a centre zero voltmeter, to ground, to monitor any shift in the electrical zero point.

BUFFERS

1 BARBITURATE pH 8.6, $\mu^1 = 0.05$ Block, Durrum, and Zweig (1955).

2 BORATE pH 8.6. Block, Durrum, and Zweig (1955).

3 (a) '0.24M BORATE' This is so called following Dawson, Elliott, Elliott, and Jones (1959), containing 74 g. boric acid and 114.5 g. sodium tetraborate in a final volume of 10 l., pH 8.55, conductivity 3.8 mmho.

(b) 0.08M BORATE GLYCINE One part of '0.24M borate' + 2 parts of distilled water. Glycine was added (6 g./l.). Final pH 8.45-8.50, conductivity 1.5 mmho.

4 (a) ALUMINIUM LACTATE, $\mu = 0.15$ (Elton and Ewart 1962) pH 3.19, conductivity 1.15 mmho.

(b) ALUMINIUM LACTATE $\mu = 0.05$, pH 3.35, conductivity 0.585 mmho.

FOR CELLULOSE ACETATE Five parts buffer no. 1 and 1 part buffer no. 2.

FOR CELLOGEL Two parts of the buffer used for cellulose acetate was mixed with 1 part of 0.2M glycine². Final pH 8.55, conductivity 2.1 mmho.

FOR IMMUNO-ELECTROPHORESIS Agar for the slides prepared by mixing equal volumes of 2% Ionagar (Oxoid, Ltd.) and buffer no. 1; for the tank, 5 parts buffer no. 1 and 1 part buffer no. 2.

FOR ELPHOR VAP APPARATUS The usual buffer was borate using 3a in the electrode vessels and borate glycine 3b in the separation chamber.

A few experiments were done with tris citrate (Bidwell *et al.*, 1966) and with aluminium lactate, buffer 4a in the electrode vessels and 4b in the separation chamber.

¹ μ = ionic strength.

²The addition of glycine was made to aid the preservation of the blood clotting factors under investigation.

POWER SOURCES

CONTROLLED POWER PACK OUTPUT ONLY

Constant current Either a laboratory made power pack or the Shandon Vokam unit was used. The Elphor VaP machine is provided with a constant current source.

Constant voltage Laboratory made power packs were used. The main A.C. supply to these packs was derived from an Advance Volstat type CV500A constant voltage transformer.

CONSTANT POTENTIAL OVER THE SEPARATION FIELD CONTROL Basically the system operates as follows. The voltage across the supporting medium is sensed by electrodes and fed into an amplifier where it is compared with a voltage preset by the operator. Information about the changes in the ratio of the two voltages and the direction of change is fed to another circuit causing a motor to drive either a continuously variable auto transformer in the mains input line; a rheostat in the positive line of the existing power pack; or the control potentiometer of the power pack itself.

It is possible to achieve the same result in a completely self-contained power pack by solely electronic methods, but it was found that a more versatile pack could be built more easily using the electromechanical system. This provided almost instantaneous correction of voltage.

The basic sensing and control system can be applied to self-contained power packs and to packs producing from a very few to 3,000 volts, the only change required being in a suitable choice of resistors in the sensing circuit to reduce the voltage to a reasonable value.

The circuit of a fully self-contained power pack for voltages between 20 and 300 will be described, followed by details of applications of the basic circuit to other power packs.

CIRCUIT

The diagram of the circuit is shown in Fig. 1, and includes facilities for warming up the pack and standby running with no potential across the output terminals, polarity reversal, and uncontrolled D.C. power facilities.

The mains input is fed through a multi-contact switch that is closed only when switched to either polarity direction or uncontrolled D.C. From this switch the supply passes to a small constantly variable auto transformer, *e.g.*, Berco Regavolt or Variac and from there to a simple half-wave rectifier and smoothing circuit. This transformer can be adjusted either manually to set the voltage across the separation field or by the automatic circuit.

In the circuit diagram the bath is shown as three resistors enclosed in a rectangle, the power terminals marked A and D, and the sensing terminals B and C. Resistors AB and CD represent the electrode and buffer chambers of the bath, and resistor BC represents the supporting medium. Points B and C are connected to the grids of two cathode followers V1 and V2. Point C may, in some cases, be only a few volts above ground, so a negative line must be provided to enable the valve to operate.

FIG. 1. Self-contained power pack. CVAT = Constantly variable auto transformer. mA = milliammeter. V = voltmeter. A and D are the power terminals to the bath. B and C are the sensing electrode terminals. V1-V4 are the cathode followers. N is the neon with potentiometer P1 across its terminals to derive the control voltage. R1 and R2 are the relays controlling the CVAT servo motor M, the sensitivity of the relay system being controlled by rheostat S.

It was found experimentally that it is not permissible to withdraw more than a few microamperes from the supporting medium as distortion of the potential field will occur. Cathode followers are used between all stages to enable connexion to be made between high and low impedance circuits.

The neon N1 is connected between the cathode of the cathode follower V3 and the negative line via its load resistor and has the 'set voltage' potentiometer P1 across it. As the neon maintains a constant potential across itself it follows that any voltage variation on V3 cathode will be transferred unchanged to the slider of P1.

V1 cathode, and P1 slider via V4 cathode follower, are taken to the bases of two transistors with relays R1 and R2 in their collectors. When V1 cathode and P1 slider are at the same potential no current will flow in the transistor bases and the relays will be de-energized. Should either the potential difference or the resistance of any part of the electrophoresis bath resistance chain alter, this change will be communicated, via the cathode followers, to the transistors which will respond by closing one of the relays. Closure of the relay will switch on the constantly variable auto transformer servo motor, and the D.C. supply to the supporting medium will increase or decrease according to which relay was energized, until equilibrium is restored and the voltage between V1 cathode and P1 slider is again zero. At this point the transistor base current will cease to flow and the relays will be de-energized, cutting off the supply to the servo motor. The sensitivity of the system can be preset by the rheostat S which controls the standing current through the transistors. In use this is adjusted to the point just below which hunting occurs.

The H.T. lines and the valve heater supplies are derived

from a conventional transformer rectifier system not shown on the circuit diagram. The voltage across the supporting medium can be read from a meter across the cathodes of V1 and V2. In a later modification the relays were dispensed with and the servo motor control was fully transistorized, as is shown in Figure 2.

FIG. 2. Sensing Unit for the Elphor VaP machine. An electrostatic voltmeter V is connected across the sensing terminals. Two neons, N1 and N2, are used to give a high enough reference voltage across potentiometer P1. It is necessary to connect the centre point of the HT power pack supplying the valves to the centre of the sensing resistance chain in order that the valves may work (for full explanation see the text).

FIG. 3. Fully transistorized servo motor control unit. For explanation see the text.

If this pack is built from new, highest quality components, including high grade meters, the component cost, excluding the chassis, lies between £30 and £40.

If the system is to be adapted to an existing power source, the D.C. power supply is omitted and the servo motor drives either a rheostat in the positive output line of the pack, or the control potentiometer of the pack itself. If high voltages are to be employed a suitable resistance chain has to be inserted in the sensing circuit to bring the potential down to a value suited to the cathode followers. It has been found useful to include manual switching facilities in the relay circuit to bring the voltage up to approximately the desired value, and also to include a current sensitive relay in the power pack output where high voltage is being used. This relay will open if the current rises above or falls below a preset value and will break the input mains to the power pack to prevent the development of dangerous currents in the event of a short circuit due to buffer leakage or air bubble formation in the apparatus.

The method of applying this principle to the Elphor VaP machine is as shown in Figure 3. A high resistance chain is strung across the sensing probes in order to reduce the voltage to a value suitable to the cathode followers. The cathode follower in the line from P1 ensures that little current is taken from this potentiometer, minimizing the voltage drop across its length. The principle of operation is, in fact, identical with that of Figure 1 with the important exception that the return lines are not connected to ground as is normal with this type of power pack, but are instead only connected to the centre

point of the resistance chain to enable the potential at this point to move in a positive or negative direction without altering the grid-cathode voltage of the sensing valves. This maintains a constant voltage between points B and C without reference to ground. If the return lines were grounded there would be a tendency to keep these points at a voltage relative to ground. It was found that on the positive line of the machine, as supplied, there was a 100 c/s ripple of 50v. amplitude at an output of 2,000v. which, when using relay control, caused a spurious hunting, cured by introducing extra smoothing across the relay holding coils. The servo motor drives the power control potentiometer of the existing pack.

MEASURING INSTRUMENTS All the power packs used were provided with conventional voltmeters and milliammeters measuring the output voltage and current of the pack. The constant potential over the separation field packs had, in addition, a voltmeter that gave a direct indication of the potential across the supporting medium or separating chamber. It was found that if continuous direct monitoring of the supporting medium was desired a high impedance ($> 10\text{M } \Omega$) was necessary. In the case of the Elphor VaP machine electrostatic meters were used.

PH METER Radiometer pH meter type 25 with scale expander. All measurements were made at 20°C .

CONDUCTIVITY METER Radiometer conductivity meter type CDM2d. All measurements were made at 20°C .

General electrical measurements were made with an Avometer Type D.

Waveform characterization was carried out with a Tekquipment oscilloscope type D31.R.

RESULTS

CELLULOSE ACETATE During the course of 40 representative runs on constant current, 0.4 mA/cm. width, the rate of migration of the albumin band varied by as much as 20% about the average. Similar results were obtained when using constant voltage at the bath input. On both forms of control the potential difference between the ends of the strip varied by as much as 50%. When on constant potential over the separation field control the variation in migration rate was $\pm 2\%$ about the average, over 100 runs. It was found that the rise in current averaged 32%, evenly spread between 17 and 50%, demonstrating that there were uncontrolled, non-repeating resistance changes taking place in the supporting medium. After the use of constant potential over the separation field was instituted, it was found that higher voltages than those quoted in the literature could be used when using solid supporting media, thus shortening the duration of the run. The reason for this is not known.

CELLOGEL Similar results were obtained to cellulose acetate, but fewer runs were carried out using constant current or constant voltage at the peak output. Constant potential over the separation field gave reproducible separations.

AGAR MICRO-IMMUNOELECTROPHORESIS Ten runs using constant current produced variable migration speeds. The potential difference over the slides was found to vary $\pm 10\%$. Twenty constant potential over the separation field runs gave identical migration speeds, and good results, with no sign of overheating as found with the constant current runs, even though the potential difference across the slides was higher (6-10 volts per cm.).

STARCH GRAIN Eight runs were carried out, all on constant potential over the separation field control inserted as a rheostat in the positive output line of a 500v. constant voltage power pack. These runs all showed an identical migration rate for the protein fractions. The current was found to rise during the first three to four hours, and then drop slowly to below the initial value. There was great variation of the current value from run to run, probably due to the varying buffer content of the supporting medium.

PEVIKON Similar results to starch grain were obtained.

ELPHOR VAP In the first few months after the installation of the machine we had to operate with unusually high mains voltage fluctuations (on occasion as much as +10 to -40% about the nominal 240v. A.C.), which were beyond the capacity of the current stabilization incorporated in the power pack supplied. Manual control of the voltage as shown on the instrument panel appeared to give the possibility of a satisfactory separation, in

spite of the theoretical disadvantages. In view of our previous experience with constant potential over the separation field, the manual control of voltage was soon replaced with automatic control. Although, for the first 30 runs the control was based on sensing between the positive H.T. line and the ground, *i.e.*, including one electrode chamber in the sensing circuit, the results were very good, giving sharp and reproducible separation patterns over periods of up to 24 hours.

For a special experiment the use of aluminium lactate buffer was required. The conductivity of this buffer was very low leading to a current well below 80 mA at 2,800v. as recorded on the instrument panel. This current is below the constant current control range on the machine as provided, but a good separation was obtained using constant potential over the separation field control on a run lasting 36 hours.

It was found that the voltage across the plates is approximately 15% below that indicated on the instrument panel of the machine, when using the buffer systems stated.

DISCUSSION

Although it has been shown by Brattsten (1955) that the effects of temperature variation are less marked with a constant current power supply it appears that the sum of all the other variable conditions in routine electrophoresis can exert a far greater effect, this being particularly the case when solid supporting media are present. Using constant potential over the separation field control these variables have no effect on the potential across the supporting medium, although the current varies during the run. As a secondary advantage mains voltage fluctuations are cancelled out and the entire range of the D.C. output can be controlled by the constant potential over the separation field device, this point being well demonstrated by the example of the use of the Elphor VaP at very low current (aluminium lactate buffer). It is also of interest that higher voltages than those in the literature may be used without deleterious heating effects. The method is also time saving in the preparation of an electrophoretic separation using solid supporting media as it is not necessary to give attention to exceedingly precise control of such variables such as the thickness and buffer content of the medium or the conductivity of the bath buffer. This point has the corollary that it is far easier to train unskilled staff to carry out electrophoresis using constant potential over the separation field.

SUMMARY

This paper describes a power supply for electrophoresis which maintains the potential across the separating field constant during the run. Experimental results for different types of electrophoresis are given. It is shown that this form of control can be applied to existing power packs.

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to a Leeds and Northrup Cleretrend 12 point recorder, provision being made for matching the photocell outputs. The recorder selects each channel in turn and prints a dot corresponding to the deflection from that channel. At the paper speed of 3 in. per hour the resultant trace is a continuous line. Channel identification is by colour and number. This type of recorder was chosen after careful consideration of those available and has proved satisfactory.

Future systems would be improved by the addition of a multipoint selector, now available for this particular recorder, which allows those channels not in use to be kept clear of the chart. In the present machine, a built-in test circuit, when switched on, applies different millivolt potentials to each channel not in use, allowing those channels to trace out steady lines and thus avoid confusion with channels carrying clot lysis information.

A photograph of the complete assembly is shown in Figure 3.

RESULTS

Figure 4 shows the good reproducibility from an actual record of two sets of six identical clots. (The clots were transferred from a water bath at 37°C. to the lysis chamber 80 min. after the addition of thrombin).

Preliminary investigations have shown that the basic instrument can be modified to record the lysis times of the clot system described by Hawkey and Stafford (1964), Nanninga, Zeller, and Maynes (1964), and Mann (1966). Provision has also been made in the circuit to allow a faster paper speed recorder to be connected to any channel to observe clotting times.

CONCLUSION

The instrument described permits the automatic simultaneous recording of the lysis time of up to 12 euglobulin clots. It can also be used for other clot lysis systems. Its usefulness is based upon its objectivity, simplicity of operation, and the economic use of the laboratory worker's time. This instrument is now commercially available from Carmanan Instrumentation Ltd, 2, Hamilton Road, Larkhall, Lanarkshire.

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CORRECTION

The legends to Figures 2 and 3 have been interchanged with their figures in the paper 'Electrophoresis using a constant potential across the separation field', by Dike and Bew (*J. clin. Path.*, **20**, 97) in the January issue of the Journal.

Determination of methaemalbumin in plasma

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Methaemalbumin has a characteristic absorption spectrum which changes on the addition of reducing agents such as dithionite (Fig. 1). This change, which is due to the formation of haemalbumin (Fairley, 1941), can be used as a basis for the measurement of methaemalbumin.

To 2 ml. of plasma (or serum) is added 1.0 ml. of phosphate buffer (1M, pH 7.4). The mixture is centrifuged for 5 min. to remove traces of fibrin and any remaining cells and the absorbance measured at 569 m μ in a spectrophotometer such as the Unicam SP500. The solution is then returned to a test-tube and a small amount (about 5 mg.) of solid sodium dithionite added. The tube is shaken gently to dissolve the dithionite and left for 5 min. to allow complete reduction of methaemalbumin. The absorbance at 569 m μ is again determined and the increase computed.

The concentration of methaemalbumin is obtained from a calibration graph. This is constructed from readings obtained with methaemalbumin solutions of known concentration (1 to 10 mg./100 ml., as haemin) prepared by dissolving haemin (British Drug Houses) in a minimum volume of 1 M NaOH and adding this immediately to a solution (4% w/v) of human serum albumin.

Measurement of absorbance before and after addition of dithionite avoids interference due to background colour or to turbidity which invalidate the spectro-

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FIG. 1. Absorption spectra of methaemalbumin (10 mg./100 ml., as haemin): A, before, and, B, after treatment with sodium dithionite.