A MODIFIED APPARATUS FOR CARRIER-FREE PREPARATIVE CONTINUOUS ELECTROPHORESIS

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INTRODUCTION

BARROLLIER, WATZKE AND GIBIAN[1] have succeeded in carrying out electrophoretic separations in a free-flowing buffer solution without sacrificing the advantages of an apparatus needing a carrier. The merits of this system have already been pointed out by HÖLZER, NOACK AND WIEK[2], HÖLZER AND WIEK[3], and SCHWARTZKOPFF AND HÖLZER[4], but non-observance of strict control of the working conditions in continuous-flow electrophoresis gives rise to considerable errors.

The direction of migration of a particle in the separating chamber is the result of the following factors: represented by vectors (a) the rate of flow of the buffer solution through the apparatus as the vertical, and (b) the electrophoretic migration at a defined field intensity and a defined pH-value as the horizontal component. If during the experiment a vector quantity is changed, the direction of migration of the particle also changes. Thus the prerequisite for good selectivity is the constancy with time of the vectors at each individual point within the chamber. If the vectors do not act with the same magnitude on the individual points within the chamber, the only result will be that the fractions do not follow a straight course, the separation sharpness remaining unaffected. However, under unfavourable conditions the separating capacity of an apparatus designed for a theoretical optimum capacity is not then attained.

In the apparatus described by BARROLLIER[1] and his co-workers a capillary flow of buffer solution passes between two water-cooled, horizontal glass plates. Strips of filter paper are positioned between the plates on both sides to act as spacers and at the same time serve to connect the troughs to the electrodes. The separating buffer is fed dropwise on to the lower, somewhat longer glass plate, is led through cellulose into the separating chamber and is delivered at the front to small strips of paper. The sample is added through holes bored in the upper glass plate. The electrode troughs are filled with buffer solution of approximately twice the concentration of the separating buffer solution and are rinsed continuously.

As the glass plates, which are 50 cm wide, are only spaced from each other by the filter paper at the edge, sagging of the top plate may occur after extensive use, thus causing distortions in the flow of the buffer solution. This phenomen of sagging was prevented by interpositioning small discs (for example of perspex or teflon);

the flow was not disturbed by this arrangement. In this way it was also possible to define the distance between the plates, which may be varied from 0.3 to 0.8 mm. At the same time the filter paper was replaced by fibre glass paper in order to prevent the absorption of proteins.

It was now found that the rate at which the buffer solution was imbibed by the small strips of paper or fibre glass paper at the front edge did not remain constant. This is explained by the fact that the small strips form a system of multiple siphons, their delivery depending on the quality of the front edge of the bottom glass plate and its wetting conditions.

Delivery of a constant quantity of buffer solution per unit time through a strip may, however, only be expected if the position of the particular strip with respect to the glass plate and the surface character of the latter remain the same. But this can only be assumed under theoretically ideal conditions. In practice, vibrations in the environment can cause the small strip to shift slightly, and pollution of the glass plate by small air bubbles cannot be avoided. The mechanism by which the liquid is conveyed depends on both the capillary system inside the strips and that between the latter and the glass surface. But as the velocity of a liquid within a capillary tube varies with the third power of the radius, a considerable disturbance is caused even by an infinitely small change. Owing to the inconstancy of flow rate of the buffer solution with time, the most essential condition for a continuously operating electrophoresis apparatus is not met.

It is also very difficult to reproduce test results exactly when new glass plates have to be used, as they are not usually of equal quality even when manufactured with the greatest care.

In order to avoid the above-mentioned disadvantages, BARROLLIER's apparatus has been modified in cooperation with the manufacturer* and the modifications are described and illustrated in Figs. 1–4 below.

APPARATUS

First of all, the two glass plates are connected to each other on all four sides by silicone rubber (Wacker-Chemie, München, Germany), which is very resistant to chemical attack. The top glass plate has at its rear end seven holes through which the buffer solution is led into the chamber (Fig. 1). At the forward end of the separating chamber the bottom glass plate is provided with 49 holes through which the buffer solution is fed to a collecting device. This device is made of Trovidur and constructed as follows: it consists of 49 cups which are connected via a system of flexible tubes to the holes in the bottom glass plate. All the cups have the same capacity. At predetermined intervals, set by means of an electric timing unit, small pins of the same standard size plunge into the cups and the buffer solution in the cups is thereby displaced and flows through a system of receptacles and flexible tubes to the collecting vessels. In order to prevent the buffer solution returning to the chamber, the bottom of each cup is provided with a small pin that functions as a non-return valve. The supply of the buffer solution to the chamber and thus to the cups is effected from a vessel located at the rear portion of the apparatus. Fresh buffer solution is constantly pumped from

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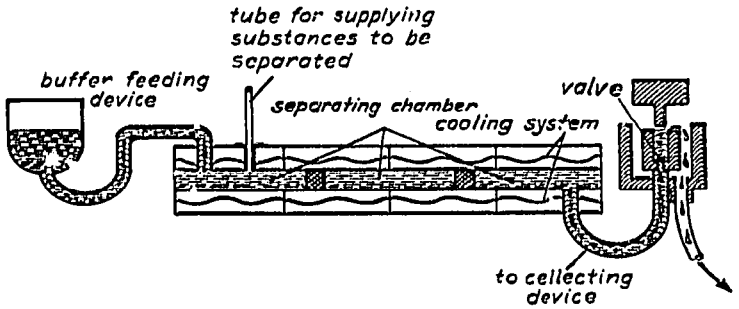

Fig. 1. Longitudinal section of the apparatus, diagrammatic.

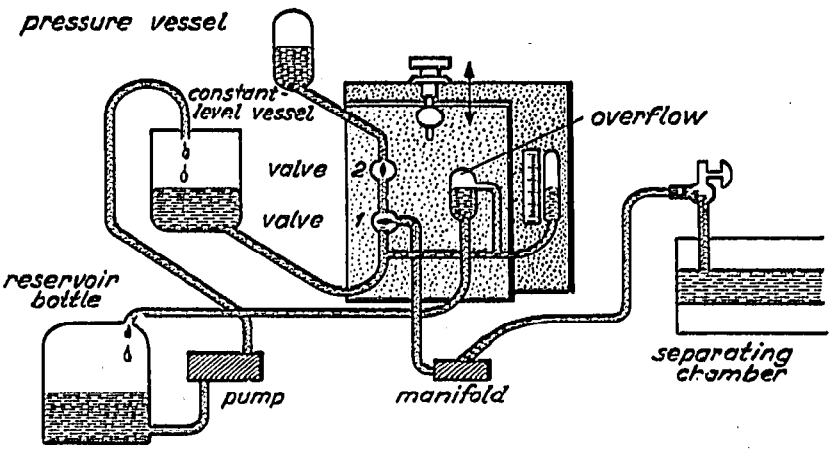

Fig. 2. Buffer supply system, partly diagrammatic.

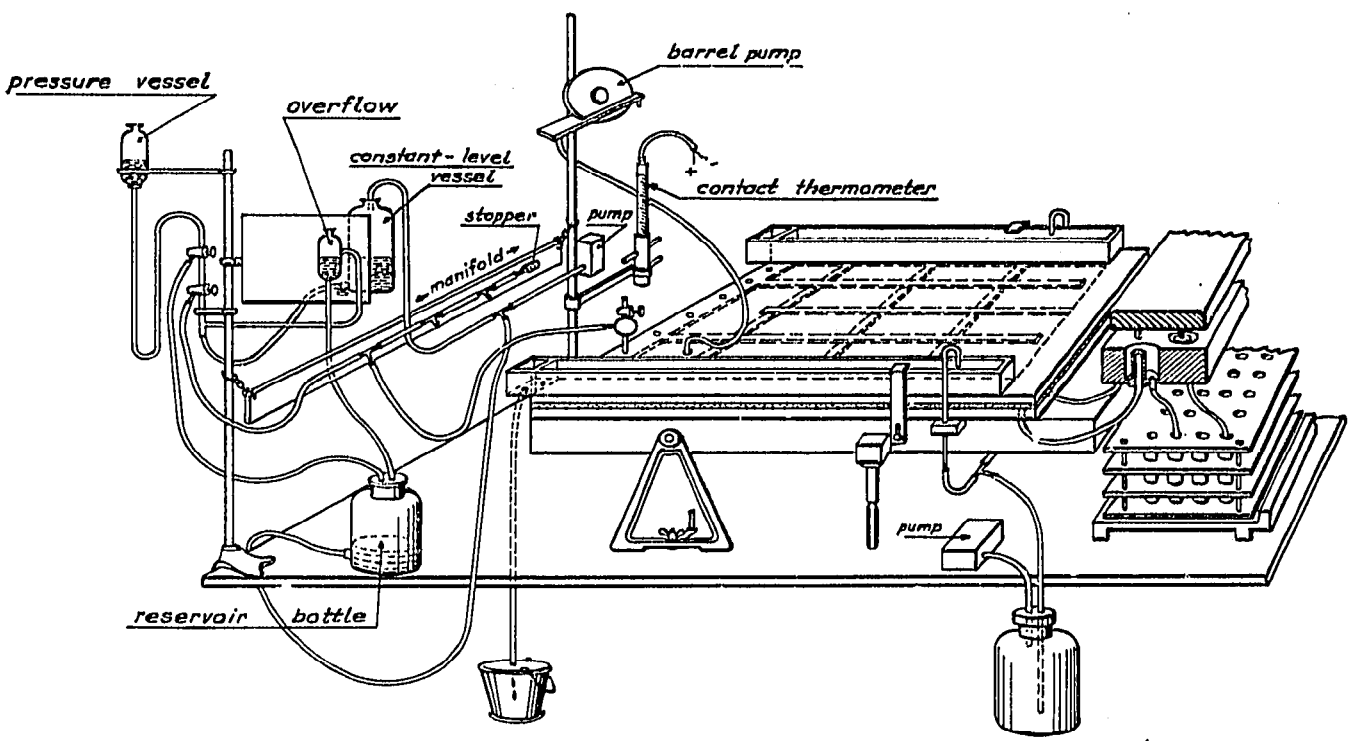

Fig. 3. Diagrammatic view of the complete apparatus. The buffer supply system is shown on the left of the illustration. The separation chamber with the two electrode troughs attached to its sides and the rinsing system for the electrode troughs are shown in the middle. The sample is fed into the chamber by the metering pump via a hose connection. The right-hand part of the illustration shows the buffer extraction system and the lines leading to the collecting vessels.

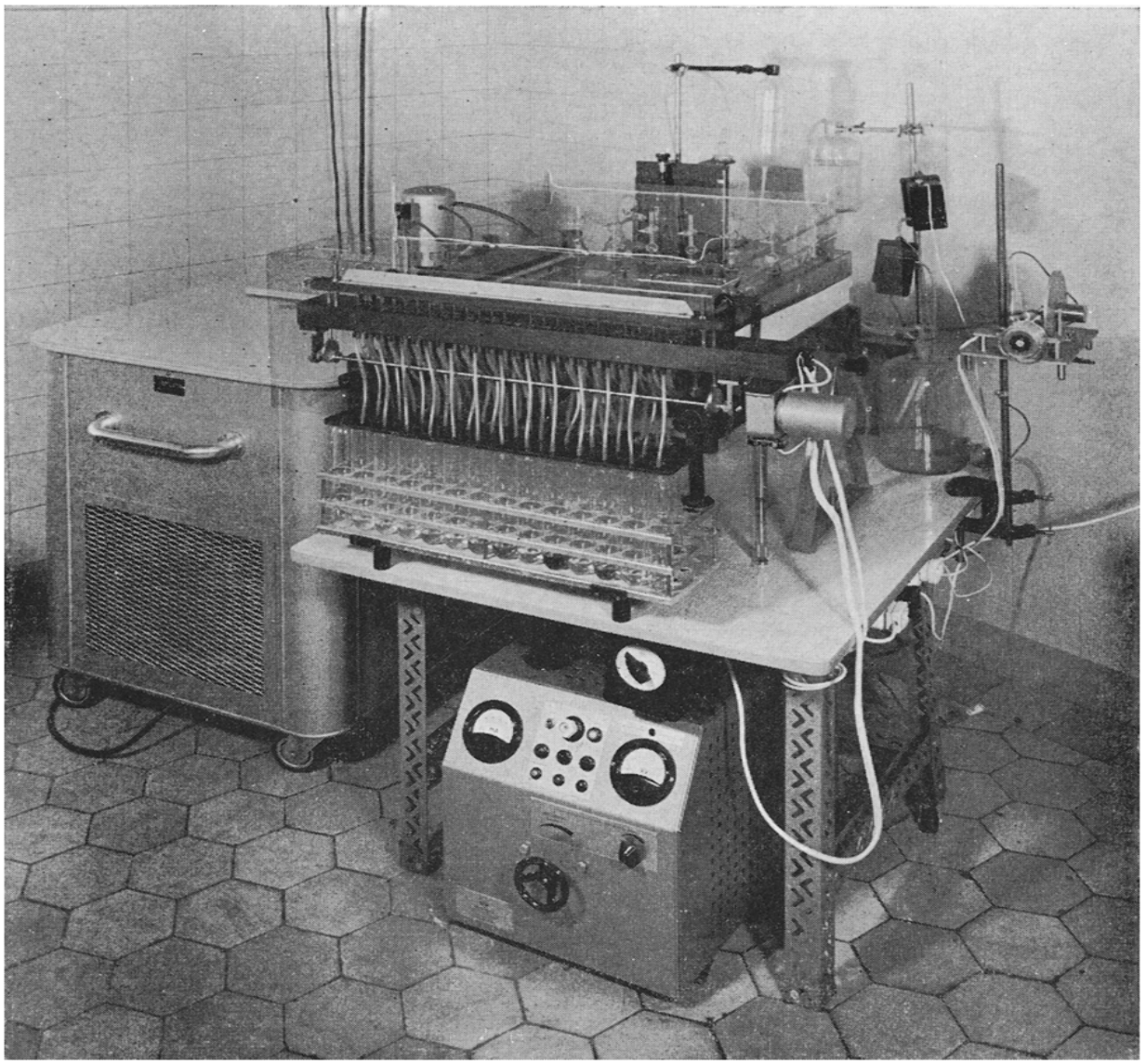

Fig. 4. A photograph showing the whole separating system. In the middle, the separation chamber, on the left the cooling aggregate, and under the table, the electric generator.

a reservoir bottle (Fig. 2), into this vessel which is provided with an overflow device. In this way the liquid level in the vessel is maintained (constant-level vessel, (see Fig. 2), but can be varied with the aid of the adjustable overflow device which in Fig. 2 is shown as the drip vessel. The constant-level vessel, the gap separating the plates and the delivery cup represent a system of communicating tubes; this ensures that the same level of liquid is also maintained in the cups. Buffer solution may be drawn from another vessel (pressure vessel) in which the liquid level is considerably higher for filling the chamber and rinsing it quickly after a test.

The troughs for the electrodes are cemented to the top plate. Holes in the top plate, which also extend through the bottoms of the troughs for the electrodes, connect the troughs with the separating chamber. Small tubes made of Trovidur are glued into these holes. To prevent the buffer solution in the troughs mixing with the buffer solution intended for the separation, the small tubes are firmly packed with

asbestos and glass wool. In this way hydrostatic effects of the buffer solutions in the troughs and the separating chamber are also avoided. Rinsing the troughs for the electrodes prevents the occurrence of polarization.

The connection of the constant-level vessel with the separating chamber is effected via a manifold having seven branches, which are seven narrow flexible tubes leading to the separating chamber. This constitutes a shunt that must have an effect on the flow of electric current. This effect was checked by measuring the resistance after filling the chamber and the system of flexible tubes with a buffer solution of veronal/veronal-sodium, ionic strength $\mu = 0.05$. In the system of tubes the resistance was 2 megohms, whereas the resistance in the chamber, which is of much greater cross-section, was as low as 8.4 kiloohms. Therefore the current flowing through the shunt can be practically neglected.

Measurements of the potential drop at the diaphragms between trough and chamber showed a drop of approximately 15% of the voltage applied to the terminals. The electric field in the chamber proved to be homogeneous.

Checks on the conductivity and the actual reaction of the buffer solution, both in the chamber and in the troughs for the electrodes, gave constant results under prolonged and varying test conditions

As in the previous arrangement a terminal voltage of 500 to 3000 volt can be applied. In the case of a chamber width of 50 cm this would correspond to 10 to 60 volt/cm. The flow rate of the buffer solution is determined by the interval at which the pins plunge into the delivery cups and by the liquid level of the latter. To control the liquid level, the constant-level vessel is connected via an ascending tube with a graduated scale so that the initial value may be determined and reproduced at any time. Thus the time for the buffer solution to pass through the system may be set for values from 20 to 360 min.

As in the unmodified apparatus, there are five holes in the top glass plate for feeding in the substance to be separated. Depending on the test conditions chosen, and on the substance to be separated, one of the holes is selected for introducing the substance into the system. In contrast to the older system, however, the holes must now be sealed to prevent the chamber from draining dry. This is achieved by fitting the holes with small pins of Trovidur which project approximately 15 mm above the glass plate. A flexible hose capable of being plugged is pulled over these pins. The substance is delivered through a metering pump of the rotary barrel type. The selectivity attainable and the constancy of the test results in this modified arrangement for carrier-free preparative continuous electrophoresis were shown by investigating the separation of human serum proteins.

EXPERIMENTAL AND RESULTS

Test conditions

Buffer: Veronal/veronal-sodium buffer solution, pH 8.6, ionic strength $\mu = 0.025$.

Terminal voltage: 1800 volts = 36 volt/cm, 100 mA.
Cooling: Inflow 0°, effluent + 3°.
Time for passage of buffer solution: 210 min.
Height of separating chamber: 0.5 mm.

Sample: 0.8 ml/h were fed into the chamber continuously. This corresponds to a

capacity of 19.25 ml serum = approx. 1.5 g protein in 24 h. The serum was undiluted and undialyzed.

Detection: The protein concentration in the collecting vessels was determined by the biuret reaction. The proportions in the fractions were found by evaluating Gauss distribution curves. The tests specified under A, C, and D were carried out in a Beckman spectrophotometer, wavelength 540 mμ, slit 0.02, whereas an Eppendorf photometer, wavelength 546 mμ, in connection with a macro trough was used for the tests listed under B.

Test A

In order to determine the order of accuracy of results under constant test conditions a normal serum (protein content 7.3 g%) was separated seven times.

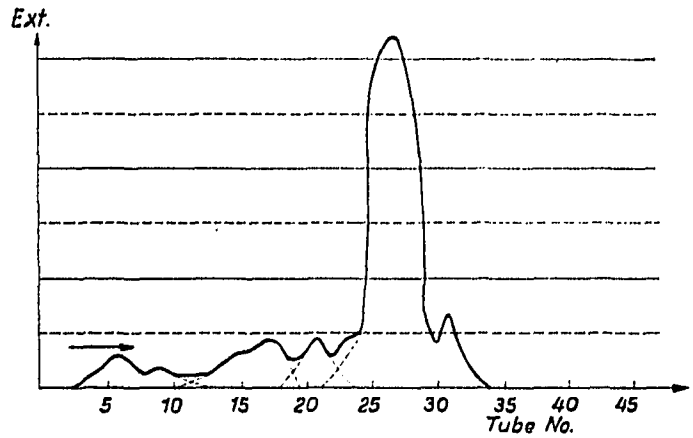

Fig. 5. Protein fractionation of a normal human serum.

Mean values (M) for the protein in the separated fractions of serum, together with the standard deviation (S.D.) and the standard error of the mean (S.E.), were calculated and are shown in Table I.

TABLE I

STATISTICAL ACCURACY OF REPEAT PROTEIN FRACTIONATIONS OF A NORMAL HUMAN SERUM

	Protein fraction			
	Albumin (%)	*α-Globulin (%)*	*β-Globulin (%)*	*γ-Globulin (%)*
M	68.47	8.61	13.14	9.77
S.D. ±	0.77	0.47	0.50	0.60
S.E.	0.29	0.18	0.19	0.23

Test B

In order to evaluate quantitative determinations of separated protein in fractions from pathological serum a range of normal values was determined by separating serum samples from 15 healthy persons. The mean (M) and the extreme values (E.V.) are given in Table II.

TABLE II

THE RANGE OF VALUES OBTAINED FOR PROTEIN FRACTIONS FROM NORMAL SERUM

	Albumin (%)	*α-Globulin* (%)	*β-Globulin* (%)	*γ-Globulin* (%)
M	63.5	10.7	11.1	14.8
E.V.	56.2–71.5	6.8–14.0	8.2–13.6	11.7–18.5

Tests C and D

Both of these separations were carried out with pathological sera which are illustrated in Figs. 6 and 7. The results are given in Table III.

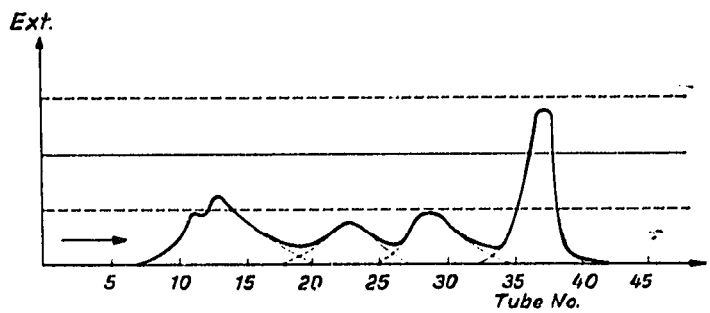

Fig. 6. Liver cirrhosis serum.

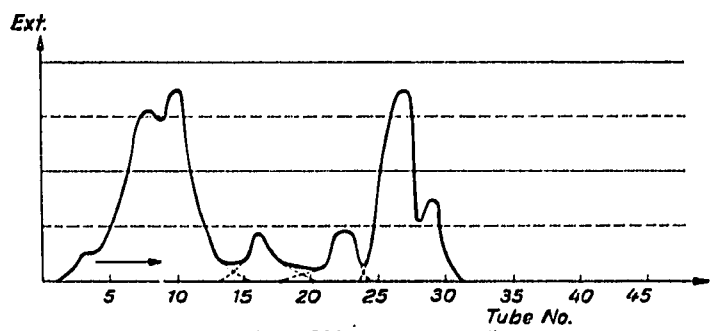

Fig. 7. Morbus WALDENSTRÖM serum.

TABLE III

PERCENTAGE PROTEIN IN FRACTIONS FROM PATHOLOGICAL SERUM SAMPLES

Sample	*Protein content* g%	*Albumin* (%)	*α-Globulin* (%)	*β-Globulin* (%)	*γ-Globulin* (%)
Liver cirrhosis serum	6.3	40.0	10.0	16.5	33.5
Morbus WALDENSTRÖM serum	9.4	29.5	6.5	6.5	57.5

DISCUSSION

The intention underlying the modification of the apparatus for carrier-free preparative continuous flow electrophoresis designed by BARROLLIER and his co-workers was to retain the separating principle but to eliminate the factors affecting the separating

process which, on account of their very limited control, gave rise to frequent trouble. To attain this end, it was necessary to redesign the entire buffer supply and extraction system.

This object seems to have been realized, judging by the separation results obtained with human serum proteins. There is satisfactory selectivity and only a very low margin of error present in the apparatus; this is comparable to the values given in the literature (RIVA[5]). The constancy of the separation results obtained is satisfactory.

In contrast to the previous design, the flow rate of the buffer solution can be mechanically adjusted and it is now possible, after modifying or cleaning of the apparatus or even use of a second apparatus, to reproduce the test conditions exactly and automatically. Automatic operation of the apparatus reduces the amount of work required to a minimum, and when starting a test the laborious filling process for the chamber, which often had to be repeated several times because of the formation of air bubbles, is no longer necessary. After the first filling, which is easily and rapidly effected by using the pressure vessel and tilting the whole chamber into a upright position, the apparatus is serviceable at all times and is capable of being put into operation with a switch.

A detailed analysis of the protein fractions present in the collecting vessels does not fall within the scope of this paper. Evaluation of the Gauss distribution curves and checks by means of the paper electrophoresis are not sufficient for qualitative analysis of the fractions and their purity yield. Further chemical, physical, and possibly serological methods are required for this purpose. Only the latter will show whether the peak before the albumin is equivalent to pre-albumin and whether the two peaks that always occur in the range of the gamma globulins are due to a true separation or only to a pseudo-fractionation.

SUMMARY

It has been shown that inconsistent results are obtained in carrier-free continuous electrophoresis according to BARROLLIER and his co-workers, in spite of all the advantages of the system. The apparatus was therefore modified. The electrophoretic separation of human serum proteins was chosen as an example to show that it is possible to achieve constant test conditions and satisfactory separation results with this modification.

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