Electrophoretic Studies of Serum Lipoproteins

I. A Description of Apparatus and Technique for their Separation in Starch Medium

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In the study of serum lipoproteins both physical methods, such as ultra-centrifugal and electrophoretical investigations, and pure chemical methods, have been applied. Serological methods have also been used.

The electrophoretical approach was chosen because of the promising results of investigations in free electrophoresis. At the time when these investigations were started (1951) the following ways were open to the study of serum lipoproteins in free electrophoresis: A) Coloring the lipids by some fat soluble dye and observing the position of the color during and after the electrophoretic separation (Bennhold¹). B) Isolating the electrophoretically separated components and analysing the fractions for various fats (Mellander², Blix *et al.*³). C) Delipidation of serum by fat extraction without protein denaturation (Blix⁴, Kunkel and Ahrens⁵), or by high speed centrifugation (Longworth *et al.*⁶), prior to the electrophoresis. The resulting decrease of some of the peaks in the electrophoretic diagram is then observed.

Since that time electrophoresis in supporting medium has been greatly developed and won widespread application, *i. a.* in the field of lipoprotein research. All the above-mentioned methods *i. e.* coloring by fat soluble dyes (Fasioli 1952⁸, Kunkel and Slater 1952⁹, Durrum *et al.* 1952⁷, Rosenberg 1952¹⁰, Swahn 1952¹¹), extracting and analysing the separated components (Kunkel and Slater⁹) and delipidation by centrifugation prior to the electrophoresis (Swahn 1953¹²) have been applied. Paper, being very easy to handle, has been the most commonly used supporting medium. Due to considerable adsorption on to the paper of β -lipoprotein (Kunkel *et al.*⁹) and low density lipoproteins (chylomicrons) (Smith *et al.* 1953¹³, Swahn¹²) in particular, paper is unfortunately not an ideal supporting medium for the study of lipoproteins. The adsorption interferes of course with the resolution of the lipid "spectrum" and with the fat extraction out of the paper. To the author's knowledge no explanation of this adsorption has been found.

In spite of these objections lipoprotein investigations by means of paper electrophoresis have yielded and confirmed earlier (Russ, Barr, and Eder

1951^{14,15}) valuable clinical informations of the lipoprotein distribution in the normal state and in various diseases with disturbed lipoprotein metabolism. (Kunkel *et al.*⁹, Malmros and Swahn 1953¹⁶, Nikkilä 1953¹⁷, Swahn¹².)

The discovery that serum lipoproteins were not being adsorbed on starch (Kunkel and Slater 1952^{9,18}) seemed to offer new possibilities for the application of electrophoresis in a supporting medium to the lipoprotein research.

The author's investigations of these possibilities started in the summer of 1953. From the beginning it was clear that a different performance of the method than that of Kunkel and Slater⁹ was desirable, for two particular reasons: First, the cutting of the starch into segments after finished separation seemed difficult to perform exactly, and secondly it would be of great advantage if one could avoid extracting the proteins from the starch.

This problem was solved by carrying out the electrophoresis containing the starch in a vertical tube and *forcing the separated components out of the starch by simple buffer flow.*

In 1950, Haglund and Tiselius¹⁹ gave a preliminary report of a similar device for zone electrophoresis in glass powder. However, these authors state: "... much more work is necessary to make the apparatus a good analytical instrument."

DESCRIPTION OF THE METHOD

Apparatus. Principally the apparatus consists of a plexiglas tube (*A* in Fig. 1) supported with a glass filter (*B*) (Jenaglass, porosity 40–90 μ) as bottom, upon which the starch column rests. It is surrounded by another plexiglas tube (*C*), serving as a cooling mantle (for further details, see Fig. 1). The top of the tube is fitted with a plate which can be screwed on to it (*D*). Centrally into this plate, a cooling glass tube (*E*) is hermetically inserted. This cooling tube is centered by a plexiglas cross (*F*), resting on the glass filter. The plate is further pierced by two holes (*G*), positions according to Fig. 1. These holes are devoted to the filling of the starch slurry, tamping and smoothing of the starch surface and the addition of buffer and protein solutions to the top of the starch (by means of a pipette). To connect the tube to the electrode vessel during electrophoresis, siphons are inserted into these two holes (Fig. 2). Finally the plate is attached to a pressure-device connection (*H*).

The filterplate is held in position by a screwed ring (*J*). For the collection of the fractions after separation, a special funnel (*K*) is screwed onto the filterholder.

For smaller tubes (up to 30 mm in diameter) the entire cooling system can be excluded. The apparatus then consists of a simple plexiglas tube supported with a glass filter as bottom as described above.

Packing of the tube. It is of utmost importance to pack the starch homogeneously throughout the tube to avoid the spreading and tailing of the zones. The packing method for starch columns described by Stein and Moore²⁰ has been found superior to all other methods tried. In this method, advantage is taken of the property of starch to absorb water while swelling. Potato starch can thus increase its volume by 55–60 % (from the anhydrous state to complete saturation). As recommended²⁰, a suspension is made of starch in butanol to which distilled water has been added to make the initial water content 30 % of the dry weight of the starch. An important point is to have all the water dissolved in the butanol before the starch is added, otherwise the starch granules will cluster.

A homogeneous suspension free of lumps is easily obtained by grinding the starch carefully with the butanol in a mortar.

Before filling the tube, the top plate with the cooling tube must be screwed on. A slightly bent glass tube is introduced into the central tube through one of the holes in the top plate. The slurry is then poured through this glass tube. In this way the slurry will flow along the walls of the plexiglas tube and incorporation of air bubbles is avoided.

Fig. 1. The principle of the apparatus. In the right top corner the top plate is seen from above to show the position of the holes.

Thereafter the holes are corked with rubber stoppers and air pressure of 4–6 cm of mercury is applied. Now the starch settles slowly for about one hour (height of starch column 30 cm). The butanol above the starch surface is then carefully withdrawn by a pipette and water is poured in. Pressure is applied again (4–6–8 cm of mercury) and the water is driven into the starch. Water is now allowed to pass through the starch for about 48 hours. During this time the starch absorbs water and swells. As pointed out earlier this swelling is of greatest importance because it creates an evenly distributed internal pressure which increases the uniformity of the intergranular spaces. After equilibration with water the desired buffer solution is driven into the starch.

The electrophoresis. To start an experiment, the buffer solution above the starch is removed to within 1 cm above the starch surface. The remaining solution is driven down

Fig. 2. Photograph of the apparatus.

Fig. 3. Principle arrangements for the electrophoresis.

by pressure. Now the protein solution, being of the same temperature as the tube system, is added. It is driven into the starch column by pressure until the very moment when the starch surface is dry. Now just enough buffer to cover the surface is carefully added and pressed down. The tube is then filled up with buffer. The starting position of the protein solution must be chosen with reference not only to the mobilities of the protein components, but also to the electroosmosis. Otherwise one or more of the components may migrate out of the starch. When *e. g.* serum proteins are to be examined in an alkaline medium, the solution must be depressed approximately one third of the distance albumin is assumed to migrate. (These distances are measured on the outside of the column and are of course relative migration distances.) This is due to the fact that the electroosmosis is directed towards the cathode and thus counteracts the migration of the proteins.

When the solution has reached its starting position, the plexiglas tube is immersed in a vessel filled with buffer solution. Thereafter two siphons are inserted through the top plate to connect the buffer solution above the starch with an electrode vessel (see Fig. 2). As during electrophoresis considerable heat is generated in these siphons due to the high current density, they have been equipped with a cooling system by inserting a cooling glass tube through their horizontal part. (The cooled solution in the horizontal part will of course sink, and in this way an effective circulation is obtained inside the siphons.) The electrode vessel is principally constructed as described by Kunkel and Tiselius²¹ and furnished with a lid, in which there are holes for the siphons.

The liquid menisci in the vessels are now adjusted to the same level (Fig. 3) so that no liquid streaming due to gravity is obtained.

An electrode is immersed in the external vessel. The electric current is now turned on and the electrophoresis started.

Fraction collection. After finished electrophoretic separation the special funnel is screwed on (with the tube still in the vessel to avoid air bubbles in the funnel). The tube is then taken out of the vessel, and a pressure of about 10 cm of mercury is applied. Fractions of constant volume are then collected, either by some volumetric device or by counting the drops. As yet this has been done manually, but an automatic fraction collector is under construction. It has been advisable to give the fractions the same volume as the applied protein solution had.

Fig. 4. Effluent diagrams from sera forced through a starch column. Each value represents one fraction (1 ml) and is expressed as percentage protein of the total amount of protein. For further explanations see text. A) Normal serum. The concentrations of the applied protein solutions were (from left to right): 7.4 %, 3.7 %, 2.5 %, 1.8 %, 1.2 %. B) Nephrotic serum. Protein concentrations: 4.3 %, 2.2 %, 1.5 %. C) Serum from a case of essential hyperlipemia. Protein concentrations: 7.8 %, 2.0 %, 1.3 %, 1.0 %.

Data for an apparatus suitable for about 4 ml of protein solution. For meaning of the letters see under the description of the apparatus.

A: Internal diameter 44 mm, external diameter 48 mm. Height 500 mm.

C: Internal diameter 64 mm, external diameter 70 mm. Height 490 mm.

E: External diameter 7 mm.

G: Diameter 14 mm.

Composition of the starch suspension to obtain a 30 cm high column: 300 g starch (calculated as anhydrous), 400 ml butanol and distilled water to make the water content 30 % of the dry weight of the starch.

EXPERIMENTAL

The protein analyses have been carried out either according to the modified biuret-reaction of Goa²¹ adapted to the "ultra-micro-scale" or to the Folin copper method²². The cholesterol has been determined by the titrimetric method of Schmidt-Thomé²⁴, which has been slightly modified. The phospholipids have been determined as inorganic phosphate, the phosphorus analyzed with ascorbic acid as reducing agent²⁵. All analyses, except those concerning the phospholipids, have been carried out in duplicate.

First of all, the possibility of forcing serum through a starch column was investigated. This investigation was carried out in tubes with a diameter of 22 mm, the starch being packed as described above. Ammonium buffer of pH 9.0 and ionic strength 0.1 was used. In each experiment 1 ml protein solution (serum, undiluted or diluted with buffer) was forced through the starch column. 1 ml fractions were collected and analysed for protein. It is of utmost importance to keep the protein zone as narrow as possible when it is forced through the starch column. Otherwise a good electrophoretic separation can not be obtained. In order to avoid spreading the zone, the protein concentration must not be too high, as is evident from Fig. 4. For normal sera as well as for a nephrotic serum the best result is achieved with protein concentrations below 1.5–2.0 %. The greatest spreading of the zone showed the milky serum from a case of essential hyperlipemia. Here the spreading is most probably due to the presence of the large lipid particles, the chylomicrons. In none of these experiments was the recovery below 95 %.

The packing of the starch. Different kinds of potato starch have been tried. The starch manufactured by Morningstar Nicol, Inc., New York, has been found superior to all others. The following ways of packing the starch were tried: A) Suspensions of starch in butanol with the water content adjusted to 10, 20, 25, 30, 35 and 40 % of the dry weight of the starch, B) Suspensions of starch in water, C) Filling the tube with starch powder during simultaneous vibrating of the tube.

The homogeneity of packing was then judged by forcing protein solutions through the starch as described above. Of these methods, the best results were obtained with a suspension of starch in butanol with a water content of 30–35 %. With this degree of packing, the starch occupies about 2/3 of the volume of the column, *i. e.* the "free space" of the starch column is 35 % of the volume.

Possible influence of increasing salt concentrations upon the spreading of the protein zone. The effect of increasing salt concentrations was investigated in ammonium buffers with pH 9.0. No influence was observed, on the spreading of the zones or on the recovery of the proteins, by varying the ionic strength from 0.05 to 0.45.

Influence of pH and of various buffer-systems upon the spreading of the protein zone. Varying the pH from 8.0 to 9.5 of the ammonium buffers did not influence the spreading or recovery of the protein zone. Results obtained with veronal buffer (pH 8.6, ionic strength 0.1) and with Michaelis' veronal-acetat buffer (pH 2.0, ionic strength 0.1) were identical with those obtained with ammonium buffers. On the other side, experiments with phosphate buffers showed unsatisfactory results with considerable spreading of the zone.

Influence of adjusting the salt concentration of the protein solution. Dialysis of the protein solutions against the buffer to be used before they were forced through the starch column, did not show any advantage.

Influence of coloring the proteins with bromophenolblue. It is a great advantage if the proteins are visible, making it possible to follow their migration. Bromophenolblue was added to serum, which was forced through the starch column. The dye itself is adsorbed on to the starch, but when it is added to serum, it is bound to the albumin and not then adsorbed. No influence was observed on the behaviour of the zone. (Because of its adsorption on to the starch the dye must not be added in excess of the serum).

The behaviour of serum lipoproteins. The fractions collected after forcing serum through a starch column were analysed for cholesterol and phospholipids. 0.70 ml of each fraction was extracted with 20 ml boiling alcohol-ether (3 : 1). The extracts were filtered, evaporated and transferred to 10 ml volumetric flasks, and made up to volume with alcohol. Of these extracts 3 ml were evaporated to dryness and extracted with petroleum-ether. The remaining alcohol extract is analysed for phospholipids. The petroleum-ether extracts were transferred to 5 ml volumetric flasks and made up to volume. Aliquots are then taken of these extracts and analysed for cholesterol.

In this way normal sera as well as pathologic sera (nephrosis, diabetes, essential hyperlipemia) have been investigated.

The recovery of the lipids have in all cases been 105–95 %. Furthermore the *per cental concentration of protein, cholesterol and phospholipids in each fraction has been almost identical.*

Extraction of lipids from the collected fractions after electrophoretic separation. From the preceding results it is evident that the protein concentration during electrophoresis should be 2 % or lower. Otherwise, due to spreading and overlapping of the zones, no good separation can be obtained. If serum has a protein concentration of between 6 and 8 % it is diluted with three parts of buffer, as a matter of course.

For the study of the lipid distribution of normal sera 1 ml is needed. This gives a volume of 4 ml after dilution with buffer. For this amount a starch column with a diameter of about 45 mm is convenient. The protein zone then measures about 8 mm initially.

Prior to the electrophoresis a few grains of bromophenolblue are added to the serum. This has two advantages: First, it is possible to follow the migration of the fastest moving component, the albumin. Secondly, if the amount of bromophenolblue is determined colorimetrically in the collected fractions after the separation, a differentiation of the albumin and the α_1 -globulin-fraction is achieved, if these are not separated.

After the electrophoresis 1 ml serum is distributed on about 35 fractions of 4 ml each, *i. e.* 140 ml totally. Due to this dilution it is technically impossible to extract the lipids

Table 1. *Extraction of cholesterol and phospholipids from serum diluted with ammonium buffer. Final volume 3 ml.*

Amount of serum per 3 ml final volume ml	Cholesterol calculated γ	Cholesterol found γ	Phospholipids calculated as γ P	Phospholipids found as γ P
0.20	24.6	24.6	10.0	9.9
		23.8		9.9
		24.6		9.0
		24.8		9.5
0.10	12.3	12.3	5.0	4.8
		12.4		4.9
		12.8		4.9
		12.2		5.1
0.05	6.15	6.3	2.5	2.4
		6.2		2.7
		6.4		2.5
		6.2		2.6
0.01	1.2	1.4	0.5	0.5
		1.5		0.5
		1.2		0.6
		1.3		0.6

with alcohol-ether without simultaneous extraction of the buffer salts. Unfortunately these buffer salts interfere in many ways during the lipid analyses.

The problem to get a lipid extract without buffer salts was solved by precipitation of the proteins with trichloroacetic acid (TCA). The precipitate is heated for half a minute on a boiling waterbath and subsequently filtered through filter paper. The filter paper with the precipitate is then extracted with 20 ml boiling alcohol-ether (3 : 1). Before this extraction, however, the TCA must be removed from the filter paper. This is obtained by rinsing twice with distilled water. If the TCA is not removed, substances interfering with the subsequent lipid analyses will be extracted out of the paper. (Pretreatment of the filter paper with alcohol-ether or 1 % TCA in alcohol-ether was not successful.)

To control the accuracy of this method, dilutions of serum were made, all having a final volume of 3 ml. After precipitation of the proteins with 1 ml 10 per cent TCA, cholesterol and phospholipids were determined as described above. As is evident from Table 1, the method gives very satisfactory results.

If bromophenolblue has been added to serum, the precipitate has a yellow color. This color still persists after the first rinsing with distilled water, but during the second rinsing the color changes to blue, indicating that the TCA has been removed.

The protein determination. The advantage of the Folin copper method is its high sensitivity. In a reaction volume of about 4.5 ml 10–700 micrograms of protein can be determined. This dilution makes it possible to use the method in case of highly turbid sera. The great disadvantage is that the amount of color is not related to the amount of nitrogen and on that account varies with different proteins. Furthermore the color reaction does not show agreement with Beer's law. (This is of course easily overcome by means of standard curves.)

The modification of the biuret reaction according to Goa adapted to the "ultra micro-scale" has the advantages of being directly related to the peptid linkage and of following Beer's law. Unfortunately the sensitivity of this method is only about one tenth of that of the Folin copper method, i. e. in a reaction volume of 0.5 ml (10)–20–400 micrograms of protein can be determined. This makes the method more liable to disturbances by turbidity.

In Fig. 5 and Table 2 data are presented from an electrophoretic experiment (normal serum, ammonium buffer of pH 9.0 and ionic strength 0.1), where the fractions have been analysed by both these methods.

Fig. 5. Electrophoresis of normal serum in ammonium buffer of pH 9.0 and ionic strength 0.1. O—O protein determined according to the modified biuret reaction. ●—● protein determined according to the Folin-copper method.

In Fig. 6 and Table 3 the results are given from an electrophoretic experiment with 1 ml of normal serum (diluted with three parts of buffer). The electrophoresis was carried out during a period of 18 hours in ammonium buffer of pH 9.0 and ionic strength 0.1, current 100 mA. Fraction volume 4 ml, of which 3 ml was precipitated with TCA, extracted with alcohol-ether and analysed for lipids as described above. The protein analyses were carried out with the modified biuret reaction.

DISCUSSION

Starch must be regarded as an almost ideal supporting medium for the study of serum lipoproteins. Except in phosphate buffer there has not been the slightest adsorption of the serum proteins as judged by the complete recovery of protein as well as lipids, when serum is forced through a starch column. Not even chylomicrons are adsorbed, as shown by Carlson and Olhagen²⁶ in a case of essential hyperlipemia. Factors like pH and increasing salt concentration have not been found to influence upon the behaviour of the

Table 2. Distribution of the serum proteins in a normal serum. Total protein 7.2 per cent. Electrophoresis carried out in ammonium buffer of pH 9.0 and ionic strength 0.1.

Fraction	Protein according to the Folin copper method		Protein according to the modified biuret reaction	
	mg/ml serum	Relative concentration in per cent.	mg/ml serum	Relative concentration in per cent.
Albumin + α_1 -globulin	37.2	55.2	45.4	63.9
α_2 -globulin	5.2	7.7	4.9	6.9
β -globulin	9.3	13.8	9.2	13.0
γ -globulin	15.8	23.3	11.5	16.2
Total	67.5		71.0

Fig. 6. Electrophoresis of normal serum in ammonium buffer of pH 9.0 and ionic strength 0.1. Upper part: ○—○ protein. ●—● color of bromophenolblue read at 595 mμ. Lower part: ○—○ cholesterol. ●—● phospholipids.

proteins when forced through the starch column. On the other side, phosphate ions seem to interact with the adsorbing power of starch.

However, there are some smaller disadvantages with starch as a supporting medium. Above all, it is the low ratio of "free space" to total volume of the column, which makes it necessary to use large columns. This will necessitate higher currents and thus increase the heat generated. Another disadvantage is

Table 3. Distribution of lipids in the electrophoretically separated lipoproteins of normal serum. (Cholesterol 178 mg/100 ml, phospholipids 172 mg/100 ml.

	Cholesterol γ / ml serum	Phospholipids * γ / ml serum	Ratio Cholesterol Phospholipids
Albumin	135	120	1.10
α ₁ -globulin	517	755	0.7
β ₁ -globulin	211	106	2.0
β ₂ -globulin	927	684	1.4
γ-globulin	123	91	1.4
Total	1 913	1 756	
Recovery	107 %	102 %	

* Lipid-P is converted to phospholipids by the factor of 25.

the electroosmotic flow, which complicates measurements of the electrophoretic mobilities. Having once been determined, however, it is easily counter-balanced by raising or lowering one of the liquid menisci in Fig. 2. The electroosmosis is more pronounced in starch than in paper and seems to be of the same magnitude as the mobility of the β -globulin in ammonium buffers of pH 9.0. There has been no evidence, that the electroosmosis disturbs the migration of the protein zones.

The choice of method for the protein determinations has been briefly discussed earlier. It can further be added that the Folin copper method gives too low values for the albumin and too high values for the γ -globulin, as is evident from Table 2 and Fig. 5.

The electrophoretic separation of the serum lipoproteins by means of this method has revealed at least 5 distinct lipoprotein components (Fig. 6). The fastest moving lipoprotein has a mobility slightly greater than the mean mobility of the albumin. The next component is the α_1 -lipoprotein, which has not separated from the albumin at the ionic strength used. At higher ionic strength, however, the α_1 -lipoprotein is separated from the albumin, which has been shown in starch electrophoresis as well as in free electrophoresis. Following this come the β_1 -lipoprotein and then the β_2 -lipoprotein. Finally there is a smaller component in the γ -globulin fraction. The separation of the lipoproteins and their behaviour at various pH and salt concentrations during electrophoresis as well as their composition and distribution in various diseases are subject to further investigations and will be discussed later on.

SUMMARY

A description and discussion is given of a method for electrophoresis in a starch column. By forcing the electrophoretically separated components out of the starch by means of buffer flow, no extraction of the proteins is needed. The usefulness of the method for the study of serum lipoproteins is shown.

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During the preparation of this article it has come to the author's knowledge that a method for electrophoresis in supporting medium, based upon the same fundamental principles, i. e. carrying out the electrophoresis in a vertical tube and forcing out the components by buffer-flow, has been developed at the Institute of Biochemistry, Uppsala, Sweden, by Drs. P. Flodin and J. Porath. Their paper will appear in Biochim. et Biophys. Acta 13 (1954) 175.

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