ELECTROPHORESIS OF LIPOPROTEINS USING PRE-STAINED SERUM

LUIZ P. RIBEIRO*

Biochemical Laboratory, Instituto Oswaldo Cruz, Rio de Janeiro (Brazil)

AND

HUGH J. McDONALD

Department of Biochemistry and Biophysics, Graduate School and Stritch School of Medicine, Loyola University, Chicago, Ill. (U.S.A.)

(Received August 7th, 1962)

McDONALD AND BERMES[1], in 1955, proposed a pre-staining technique for the determination of serum lipoproteins by paper electrophoresis, in which a saturated solution of SBB or AcSBB** in 95 % ethanol was used. The acceptance of this method grew rapidly and later modifications were introduced by WILCOX *et al.*[2] and by KANABROCKI *et al.*[3], in attempts to simplify the original method. However, the use of ethanol in these methods was said to present some possible limitations since the lipoprotein complex may be sensitive to even small amounts of this solvent[4].

Thus, SOLINAS *et al.*[4] proposed a method in which diacetin was used as solvent for SBB. Later, LARKEY AND BELKO[5] used a mixture of petroleum ether–ethyl alcohol (1:4) and ZAKELJ AND GROS[6] proposed the use of a mixture of dioxane and ethylene glycol. Further, other systems have been tried out with greater or lesser success. Among these, a system containing ethyl acetate and propylene glycol was successfully used by McDONALD *et al.*[7,8], in the chromatographic separation of lipoproteins.

In an attempt to overcome some of the difficulties found in this type of determination, solvents other than ethyl alcohol and diacetin were re-investigated by McDONALD AND RIBEIRO[9]. This investigation showed that propylene and ethylene glycol possessed some inherent advantages as compared to ethyl alcohol and other solvents.

Due to the interest shown in these pre-staining methods, we decided to undertake a more detailed study of the use of propylene and ethylene glycol as solvents for SBB in the pre-staining of lipoproteins.

EXPERIMENTAL

Sudan Black B solutions

Preliminary experiments showed that propylene glycol solutions of SBB always contained more dissolved dye than those prepared with ethylene glycol, as shown by spectrophotometric measurements.

* Fellow from the National Research Council of Brazil.

** The following abbreviations will be used: SBB = Sudan Black B, AcSBB = Acetylated Sudan Black B.

Using the technique previously described by MCDONALD AND RIBEIRO[9] it was also verified that AcSBB always gave somewhat sharper differentiation between the lipoprotein zones. Therefore, all subsequent experiments were performed using a saturated solution of AcSBB in propylene glycol. The saturated solution was prepared by heating 10 ml of propylene glycol (Eastman Kodak Co., Rochester, N.Y.) to 100–110° and 0.1 g of AcSBB, prepared according to CASSELMAN[10] from SBB (Hartman-Leddon Co., Philadelphia, Pa., certification No. Czb-5), was added to the hot solvent, with thorough stirring for 5 min. The solution was filtered hot on Whatman No. 2 filter paper, cooled and re-filtered on the same kind of paper.

Care must be taken not to exceed 110° to avoid the formation of a useless gelatinous mixture. The solution thus prepared was found to be stable for at least one month, when kept in a tightly-closed container in the dark.

Paper electrophoresis

The paper electrophoresis apparatus used for these experiments was of the horizontal type (Buchler Instruments, Inc., New York City) and all separations were performed using a veronal–Na veronal buffer having a pH of 8.6 and an ionic strength of 0.05. Of the several types of paper used, we found that Whatman No. 3MM and Macherey and Nagel No. 2214ff were the most suitable for this type of experiment. However, it seemed that Macherey and Nagel No. 2214ff offered some slight advantages since the bands appeared somewhat more clearly separated when this paper was used.

The ionograms were scanned with the aid of the Photovolt Transmission Densitometer Model 525 (Photovolt Corp., New York City) and the measurements made at 595 mμ. Quantitation of the ionograms was made by planimetry.

RESULTS AND DISCUSSION

Amount of dye solution to be added to the serum

Since excess solvent may cause alterations in the lipoprotein patterns the amount of dye solution to be added to the serum should be determined carefully. This determination was carried out by adding slowly, with constant, gentle stirring, increasing amounts of the saturated solution of AcSBB to the same volume of identical serum samples (0.5 ml) placed in small centrifuge tubes.

The serum–dye mixture was allowed to stand at room temperature for 45 min and then centrifuged for 15 min. Aliquots of 20 μl of the supernatant were used for the electrophoretic separations using a potential gradient of 8 V/cm for 2 h. After fractionation, the strips were dried at 50° in the dark and scanned. Table I shows the results of these experiments.

The results illustrated in Table I show that from No. 1 to No. 3 there is a linear increase of the areas of the fractions with the amount of dye solution added to the serum. This is evident from the fact that the values of the β/α-ratio remain constant. However, this does not hold for the values in strip No. 4.

Since the α- and β-lipoprotein zones are decreased in strip No. 4 and the chylomicrons are increased, it is possible that some denaturation occurred at this point due to excess solvent added to the serum. These facts are visualized better in Fig. 1 and suggest that pre-staining should be carried out by adding less than 0.1 ml of

TABLE I

AREA OF THE LIPOPROTEIN ZONES AFTER ELECTROPHORESIS OF SERUM PRE-STAINED WITH INCREASING AMOUNTS OF DYE SOLUTION

Strip No.	*Amount of dye solution**	*Area (cm²)*				*β/α-ratio*
		Chylo-microns	*β-Lipo-proteins*	*α-Lipo-proteins*	*Total area*	
1	0.025	1.05	2.50	0.95	4.50	2.7
2	0.050	2.25	4.50	1.65	8.40	2.7
3	0.075	3.25	6.85	2.55	12.65	2.7
4	0.100	5.05	6.25	2.45	13.75	2.5

* ml of dye solution added to 0.5 ml of serum.

dye solution to 0.5 ml of serum. The point where 1 volume of dye solution is added to 10 volumes of serum was selected as the most convenient proportion for pre-staining lipoproteins.

Time of incubation

The time of incubation of the serum sample with the dye solution was determined by incubating aliquots of 0.5 ml of the same serum sample with 0.05 ml of the saturated solution of AcSBB for different periods of time. After the incubation period was over, all tubes were centrifuged for 15 min and samples of 20 μl used for the electrophoretic fractionation. Separations were carried out for 2 h using a potential gradient of 10 V/cm.

After electrophoresis, the strips were dried in the dark at room temperature and scanned. The results obtained are presented in Table II.

The serum and dye solution mixture was also incubated at 37° for 30 and 40 min, respectively. However, the results failed to show any improvement when the incubation mixture is kept at 37°, as proposed by LARKEY AND BELKO[5]. Since increasing

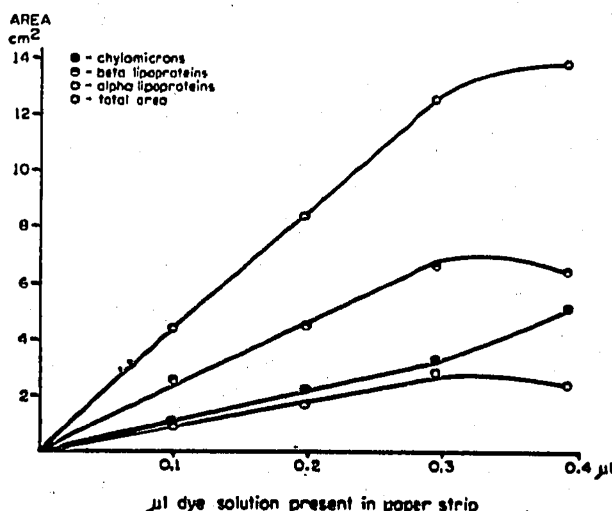

Fig. 1. Effect of the amount of dye solution added to the serum on the color intensity of the lipid zones.

TABLE II

AREA OF LIPOPROTEIN ZONES AFTER ELECTROPHORESIS OF SERUM INCUBATED WITH DYE SOLUTION FOR DIFFERENT PERIODS OF TIME

Strip No.	*Time of incubation* (min)*	*Area (cm²)*		*β/α ratio*
		α-Lipoprotein	*β-Lipoprotein*	
1	5	0.50	2.61	5.22
2	10	0.55	2.60	5.00
3	25	0.50	2.70	5.40
4	35	0.84	3.30	3.93
5	45	0.83	3.28	3.95
6	60	0.85	3.31	3.89

* All tubes incubated at 25°.

the temperature may also alter the protein–lipid complex because of its sensitivity to heat, 25° was selected as the most suitable temperature for accomplishing pre-staining of serum lipoproteins.

Amount of stained serum added to the paper

After establishing the previously discussed conditions it became necessary to check the dependence of the amount of pre-stained serum in the strip with Beer's law. This dependence was studied with a serum sample pre-stained by adding 0.05 ml of the saturated solution of AcSBB to 0.5 ml of serum. After 45 min at room temperature the tubes were centrifuged and aliquots of 5 μl to 50 μl were used for the electrophoretic separations. These were carried out on Whatman No. 3MM filter paper, using a potential gradient of 7.7 V/cm for 2.5 h.

The strips were then dried, scanned, and the areas found for α- and β-lipoproteins were plotted on a graph against the volume of pre-stained serum applied to the paper strip, as shown in Fig. 2.

Fig. 2 clearly shows that there is a linear increase of the area between the points

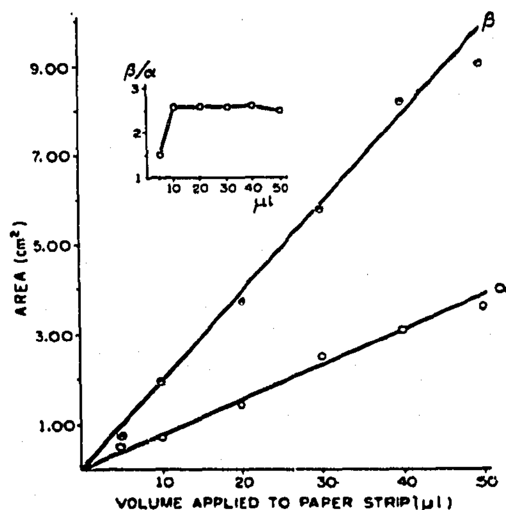

Fig. 2. Variation of the area of lipoprotein fractions with the volume of pre-stained serum applied to the paper strip.

where 10 μl and 40 μl of pre-stained serum were applied to the paper strips. The β/α-ratio was practically constant throughout the range where linearity holds. Since the linear increase follows a straight line passing through the origin, the β/α-ratio is given by the ratio between the slopes of the lines representative of the β- and α-lipoproteins, respectively, and therefore there is an agreement with Beer's law.

We have selected 20 μl as the optimal volume to be used in further experiments since this point falls within the linearity range and yet is sufficiently distant from the limits of this range.

Stability of the color

The color of the ionograms of serum lipoproteins pre-stained with either SBB or AcSBB is sensitive to light[3,11]. In order to evaluate the effect of light on the stained lipoproteins, several samples of the same serum were pre-stained using the conditions selected in the preceding experiments. Electrophoresis was carried out applying 20 μl of each sample to Macherey and Nagel No. 2214ff filter paper strips under a potential gradient of 8 V/cm for 2 h. The strips were dried at 50° in the dark and immediately scanned. They were then separated into two groups of 3 strips each. One set was left exposed to the ordinary light (daylight and artificial light) of the laboratory. Both sets were scanned periodically.

In all strips the areas of the individual fractions were measured and the results, expressed as per cent of original readings, show immediately the amount of change which occurred. The results are in Tables III and IV.

TABLE III

PER CENT OF INITIAL AREA FOR LIPOPROTEINS IN THE STRIPS KEPT IN THE DARK

Fraction	*Hours kept in the dark*					
	0	*1.5*	*3*	*17.5*	*25.5*	*50*
α-Lipoproteins	100	102.4	102.4	98.4	101.2	98.4
β-Lipoproteins	100	101.0	101.8	96.6	99.2	98.4
Chylomicrons	100	98.6	103.5	102.1	96.5	96.5
Total area	100	100.7	102.5	98.8	98.9	97.8

TABLE IV

PER CENT OF INITIAL AREA FOR LIPOPROTEINS IN THE STRIPS EXPOSED TO LIGHT

Fraction	*Hours exposed to light*				
	0	*1*	*2*	*17*	*25*
α-Lipoproteins	100	94.6	79.2	72.1	69.5
β-Lipoproteins	100	99.2	89.6	80.2	64.5
Chylomicrons	100	87.7	79.4	81.1	74.1
Total area	100	94.7	83.8	78.2	68.5

The results of these experiments indicate that the color of stained lipoproteins is quite sensitive to light. However, this color seemed stable for about 50 h, when the strips were kept in the dark. There is also evidence that light is not the sole cause for

fading of SBB bound to, or dissolved in, human serum lipoproteins. McDONALD AND BANASZAK[11] showed that there is a difference in the rate of fading of SBB bound to high-density and low-density lipoproteins when they are treated with 1 % hydrogen peroxide. The fading of SBB bound to the low-density lipoproteins occurs at a much faster rate than that bound to high-density lipoproteins. However, it was also observed that the addition of serum proteins inhibits the fading of SBB which is bound to ultracentrifugally-prepared lipoproteins.

These findings seem to suggest that fading of color in ionograms of lipoproteins pre-stained with AcSBB or SBB as described, is of a complex nature and that an oxidative reaction (probably catalyzed by light) plays an important role in the process. However, since pre-staining for electrophoresis is accomplished in the whole serum, fading is less of a problem due to the presence of serum proteins, and especially when the strips are protected from light, than that observed with ultracentrifugally-prepared lipoproteins.

Proposed technique

The results so far discussed show that a simple technique may be proposed for the determination of serum lipoproteins by paper electrophoresis, using pre-stained serum. Pre-staining can be achieved with a saturated solution of AcSBB in propylene glycol by adding 1 volume of the dye solution to 10 volumes of serum. After 45 min at room temperature the mixture is centrifuged and the supernatant used for the electrophoretic separations.

The pre-stained serum (20 μl) is added as a thin streak across the width of Macherey and Nagel No. 2214ff paper strips (2.5 cm × 33 cm in our apparatus) midway between the ends. A potential gradient of 8 V/cm is applied for 2 h, at 25°; it developed a current of 1.5 mA/strip at the end of the run, under our experimental conditions. Separations are carried out using veronal buffer with a pH of 8.6 and an ionic strength of 0.05. After completion of the run (carried out in the dark) the strips are removed and dried immediately in a horizontal position, at 50°, in the dark, and scanned. Duplicate runs of individual samples are always made.

Reproducibility and error of the method

Seven samples of the same serum were individually pre-stained and run in two separate sets, on two consecutive days. The electrophoresis was performed with the recommendations described in the preceding section, using Macherey and Nagel No. 2214ff filter paper strips. The usual 8 V/cm potential gradient was used for a period of 2 h.

When the electrophoretic separation was completed, the strips were dried in the

TABLE V

COEFFICIENT OF VARIATION FOR THE VALUES OF THE DIFFERENT LIPOPROTEIN FRACTIONS, EXPRESSED AS PERCENTAGES

Fraction	Variation (%)
α-Lipoproteins	± 3.0
β-Lipoproteins	± 2.6
Chylomicrons	± 3.0
β/α-ratio	± 5.4

dark, and scanned. After planimetry of the areas, the results were expressed as percentages of the total area and the standard deviations calculated. From this data the coefficient of variation for each fraction was calculated. This permitted a computation of the error of the method (as percentages) as shown in Table V.

The data in Table V show that the results obtained by the method described are quite reproducible and that they are within ± 3.0 % for each individual fraction. The method, which is simple to perform, time-saving and inexpensive, can therefore be considered to have a good precision and reproducibility.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. ADOLF RADMACHER of the Macherey, Nagel and Co., Düren, Germany, for kindly providing the samples of filter paper Macherey and Nagel No. 2214ff used in this investigation. The project, in its earlier phases, was supported in part by a grant-in-aid from the Chicago Heart Association.

SUMMARY

A simple method for the paper electrophoretic determination of serum lipoproteins, pre-stained with a saturated solution of AcSBB in propylene glycol, is described. Pre-staining is carried out by adding 1 volume of the dye solution to 10 volumes of serum. Electrophoresis is performed on Macherey and Nagel No. 2214ff filter paper in veronal buffer, pH 8.6, ionic strength of 0.05. A potential gradient of 8 V/cm is used for a period of 2 h.

The conditions for pre-staining are discussed and the influence of light and other factors are taken into consideration. The method is quite simple, inexpensive, and time-saving, showing a reproducibility of results which is within ± 3.0 % for each individual fraction.

REFERENCES

1 H. J. McDONALD AND E. W. BERMES, JR., *Biochim. Biophys. Acta*, 17 (1955) 290.
2 A. A. WILCOX, P. T. WERTLAKE, M. I. HALEY AND J. E. PETERSON, *Proc. Soc. Exptl. Biol. Med.*, 98 (1958) 718.
3 E. L. KANABROCKI, E. KAPLAN, D. S. KINNORY, A. A. IMPERATO, J. E. BERRY AND L. A. BAKER, *Clin. Chem.*, 4 (1958) 382.
4 P. SOLINAS, R. BETTI AND E. F. P. DI LEO, *Clin. Chim. Acta*, 2 (1957) 586.
5 B. J. LARKEY AND J. S. BELKO, *Clin. Chem.*, 5 (1959) 566.
6 A. ZAKELJ AND M. GROS, *Clin. Chim. Acta*, 5 (1960) 947.
7 H. J. McDONALD AND J. Q. KISSANE, *Anal. Biochem.*, 1 (1960) 178.
8 H. J. McDONALD, L. J. BANASZAK AND J. Q. KISSANE, *Anal. Biochem.*, 1 (1960) 44.
9 H. J. McDONALD AND L. P. RIBEIRO, *Clin. Chim. Acta*, 4 (1959) 458.
10 W. G. B. CASSELMAN, *Quart. J. Microscop. Sci.*, 95 (1954) 321.
11 H. J. McDONALD AND L. J. BANASZAK, *Clin. Chim. Acta*, 6 (1961) 25.