SEPARATION OF RABBIT PLASMA PROTEINS BY ZONE ELECTROPHORESIS ON "PEVIKON C. 870"

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INTRODUCTION

The use of PVK**, a powdered co-polymer of polyvinyl chloride and polyvinyl acetate, for separation purposes has been proposed by MÜLLER-EBERHARD[1].

This paper reports on the degree of separation achieved using this material in different buffers, as studied by starch gel[2,3], paper[4], cellulose acetate[5] and immuno-electrophoresis[6]. Further purification of some fractions was attempted using starch-gel electrophoresis followed by elution into PVK[7].

Techniques were also devised to facilitate the location of protein bands in the PVK.

METHODS

Rabbit plasma proteins were obtained by collecting blood in a solution of the di-sodium salt of EDTA (JACOBSSON[8]) and dialysing against the following buffers for 24–48 hours.

Barbital buffer, pH 8.6

Sodium barbital	0.075 *M*
Diethyl-barbituric acid	0.015 *M*
Na_2-EDTA·$2H_2O$	0.0017 *M*

Tris buffer, pH 8.8

Tris-(hydroxymethyl)-amino-methane	0.35 *M*
Citric acid	0.03 *M*
EDTA	0.002 *M*

PVK electrophoresis

13 ml clarified plasma containing 0.9–1.0 g proteins were injected from a syringe into a slit cut 4 to 6 cm from the cathode end of a PVK block measuring 36 × 19 × 1.5 cm. The protein sample did not reach to the bottom nor extend to the edges of the block, which was elevated 6–7 cm above the level of the buffer. 1.2 l buffer was used in each bi-compartmented vessel, connections to the block being established by three thicknesses of surgical lint. Electrophoresis was performed at + 3°, the system being en-

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** Abbreviations: PVK = Pevikon C-870; EDTA = ethylenediaminetetraacetic acid; CM-cellulose = carboxymethyl-cellulose.

closed in a thin polythene sheet. A potential gradient of 3.7–3.9 V/cm giving rise to a ucrrent of 93–96 mA was maintained for 33–36 h, during which time the albumin migrated to a position at least 25 cm from the origin. The buffer in the electrode cells was changed three or four times and the progress of electrophoresis checked either by scanning the block with a monitor (if ^{131}I-labelled proteins were used) or by visual examination of the surface of the block in ultraviolet light.

Identification of proteins

At the end of the electrophoresis the block was dried in a current of warm air and autoradiographed after interposing a polythene sheet between the X-ray film and the block. Occasionally a longitudinal strip of PVK was cut off and the proteins contained in 1-cm segments eluted and estimated colorimetrically. The PVK block was segmented on the basis of autoradiographic appearance and of colour. The protein fractions in buffer recovered from the PVK by filtration through a Büchner funnel, were centrifuged and concentrated by pressure dialysis. The buffer used for the separation, or 0.15 *M* sodium chloride, was used for the elution. After a further clarification the fractions were tested by electrophoresis on starch gel according to SMITHIES[2] and POULIK[3], on paper in a DURRUM[4] apparatus using barbital buffer 0.05 μ pH 8.6 and on cellulose acetate according to KOHN[5]. Immunoelectrophoresis was carried out according to SCHEIDEGGER's micromethod[6] using a goat anti-rabbit serum kindly supplied by Dr. HUMPHREY.

For further purification of several fractions large scale horizontal starch gel electrophoresis in Tris buffer[3] was carried out using a 21 × 19 × 1.2 cm starch gel slab and inserting 80–90 mg protein in 1–1.1 ml taken up in PVK or Ford's A4 paper. Protein bands were located by staining a superficial slice of the gel and by autoradiography, after which selected segments were transferred to a PVK bed for elution[7].

The proteins were estimated either according to GORNALL *et al.*[9] or by measuring the absorption at 284 mμ[10]. Plasma proteins were labelled with ^{131}I according to MCFARLANE[11]. Orosomucoid was localised by comparing its mobility with that of a preparation obtained by the method of WEIMER *et al.*[12–13]. For purposes of comparison, rabbit albumin was prepared using CM-cellulose[14–16] and rabbit haemoglobin according to NYMAN[17]. Ceruloplasmin was localised with *p*-phenylenediamine according to MORELL AND SCHEINBERG[18] or with dianisidine according to OWEN AND SMITH[19]. ^{59}Fe was used to locate transferrin (GIBLETT *et al.*[20]) and haptoglobin was located with the benzidine reagent (FRANKLIN AND QUASTEL[21]) or by dianisidine staining[19]. Lipoproteins were stained with Sudan Black B (FINE AND BURSTEIN[22]) and other proteins with Amido Black[2] or Nigrosine (0.02 %).

RESULTS

1. *Separation using Barbital buffer*

Excellent reproducibility was obtained (*cf.* Fig. 1). Albumin as seen under ultraviolet light appeared as a mauve band, α_1-globulins as a yellow band, while α_2- and β-globulins were brownish, which was also the case for free haemoglobin, travelling in the β–γ region. In transmitted visible light only free haemoglobin, β-, α_2-globulins and albumin were visible, the last mentioned being difficult to detect. The plasma proteins were all labelled to some extent with ^{131}I, whereas ^{59}Fe attached itself only to β-

globulins. Thus, by a combination of visible and ultraviolet inspection, localisation of proteins other than fibrinogen and γ-globulin was possible, and these two could be dealt with by autoradiography.

The bands were then cut out, eluted, concentrated by pressure dialysis and tested for purity in different media. γ-Globulin was shown to be free of other proteins by

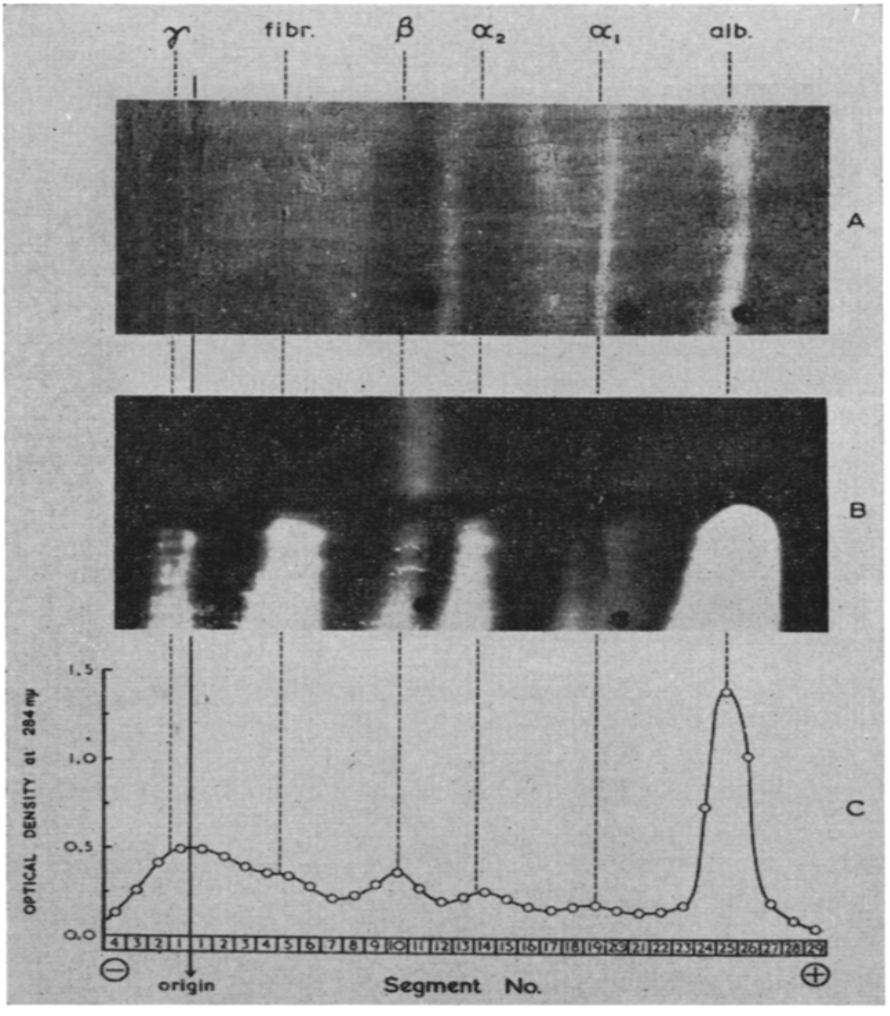

Fig. 1. (A) Ultraviolet fluorescence photograph of PVK block after the electrophoretic separation of serum proteins. (B) Autoradiograph of the same block, one half of which received ^{59}Fe-plasma and the other ^{131}I-plasma. (C) Pattern obtained by optical density measurements on plasma fractions obtained by elution of PVK segments (6 × 1 × 1.5 cm).

electrophoresis on starch gel (Fig. 2), and on cellulose acetate and by immunoelectrophoresis. Fibrinogen was contaminated with γ-globulin and the eluted radioactivity was 50–60 % clottable using JACOBSSON's method[8].

Although α_1-, α_2- and β-globulins showed apparent homogeneity on paper they appeared to be very complex on starch gel (Fig. 2). α_1-Globulins were found to be better separated using POULIK's modification[3] of SMITHIES's method than by employing vertical starch gel electrophoresis[2]. On the other hand β-lipoprotein and a slow α_1-protein were better resolved if the vertical system was used. α_1-Globulins were not contaminated with albumin and this was confirmed by autoradiography and by im-

munoelectrophoresis (Fig. 3). Albumin showed two components on starch gel (Fig. 2) with a trace of a third, while on cellulose acetate and on paper electrophoresis it gave only one sharp band.

The recoveries of the proteins expressed as percentages of the original radioactivity in PVK segments were between 90 and 95 %. However, the actual amounts

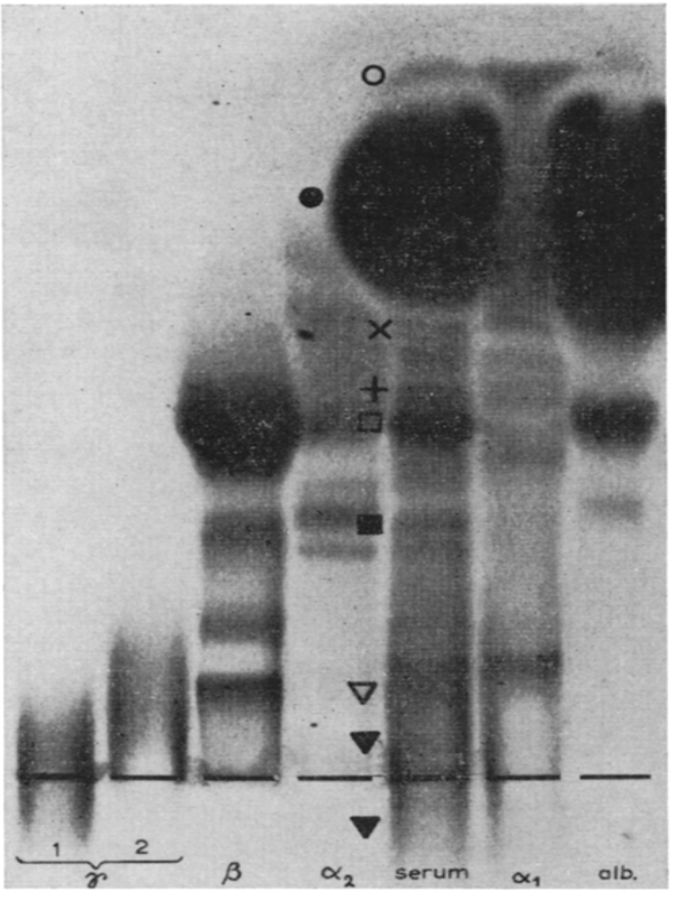

Fig. 2. Stained starch gel electrophoretic patterns of fractions obtained by PVK zone electrophoresis of rabbit serum in barbital buffer. γ-Globulin was divided arbitrarily into fractions which migrated cathodically (1) and anodically (2) in PVK. The symbols have the following meanings: ○ = orosomucoid; ● = albumin; × = post-albumins; + = ceruloplasmin; □ = transferrin; ■ = haptoglobin; ▽ = β-lipoprotein; ▼ = γ-globulin.

recovered were between 60 and 70 % when only the centres of the bands were selected in order to obtain greater homogeneity. The protein recoveries in one experiment are reported in Table I.

The recovery was 66 % for the total protein and 78 % for the albumin since albumin represented 62 % of the whole serum protein.

2. *Separation using Tris buffer*

Tris buffer was tried since it has been reported to improve the separation of plasma proteins in paper, cellulose acetate (ARONSSON AND GRONWALL[23]), and starch gel (POULIK[3]). No electro-osmotic cathodic flow was noticeable and satisfactory separations (*cf.* Fig. 4) were obtained.

γ-Globulin was homogeneous; β-, α_2- and α_1-globulins appeared to be complex fractions which were not better resolved than when barbital buffer was used. Albumin, in two experiments, gave practically only one component on starch gel, and also on

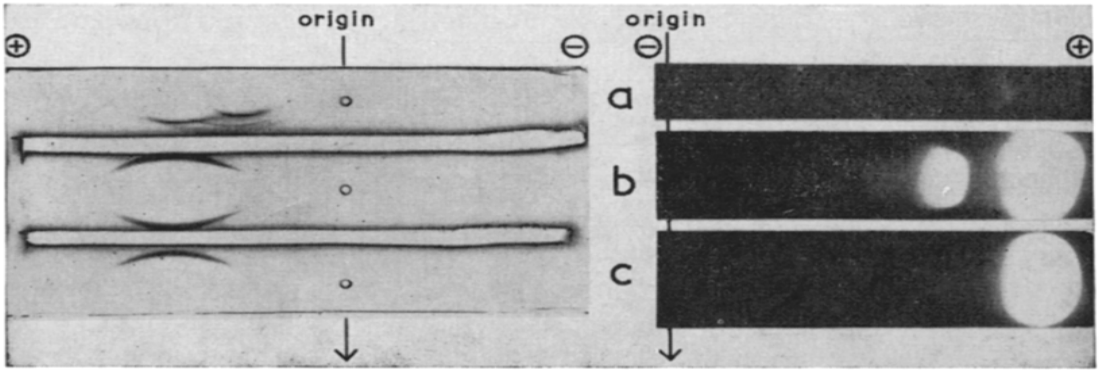

Fig. 3. Immunoelectrophoresis (left) and starch-gel autoradiographs (right) of ^{131}I-labelled PVK fractions, *viz.* (a) α_1-globulins, (b) albumin, (c) albumin (main component) after being separated by starch gel electrophoresis. In the troughs goat anti-rabbit serum.

paper it gave an unusually sharp band. The separation of α_1-globulins and albumin was satisfactory and no traces of highly-labelled albumin were present in the α_1-globulin autoradiographs.

3. *Nature of albumin contaminants*

Some experiments were devised to elucidate the nature of the minor components visible when albumin obtained by PVK block electrophoresis was run in starch gel.

These components do not represent metastable polymers since the isolated components could be re-run with unchanged electrophoretic mobilities. In Fig. 5 albumin, from the preliminary PVK run, was electrophoretically separated twice at right angles in starch gel using either borate[2] or Tris[3] buffers with identical results. Also no changes were observed in starch gel containing 8 *M* urea (SMITHIES AND CONNELL[24]) (Fig. 6).

Reducing agents were also used to break down hypothetical disulphide bonds (DEUTSCH AND MORTON[25]). Cysteine (0.05 *M*) and 2-mercaptoethanol (0.06 *M*) used

TABLE I

RECOVERY OF PROTEINS (IN mg) AFTER SEPARATION OF RABBIT SERUM PROTEINS (940 mg) BY PVK BLOCK ELECTROPHORESIS

Albumin	*Globulins*			
	α_1	α_2	β	γ
457	25*	22	44	69

separately with a phosphate buffer pH 7.5, 0.1 μ, both reduced the proportion of components slower than albumin (Fig. 6).

Only one precipitation line was demonstrable by immunoelectrophoresis for both the multi-component and the isolated one-component albumin (Fig. 3).

Other tests also excluded the possibilities that the slow components could be due to the iodination technique, ^{131}I self-irradiation or to ultraviolet irradiation.

4. *Starch gel electrophoretic separation of β-globulins*

The β-globulins obtained from the preliminary PVK run appeared to be mainly transferrin and a β-lipoprotein with minor unidentified fractions. In six rabbits so far examined only one transferrin could be demonstrated autoradiographically using ^{59}Fe.

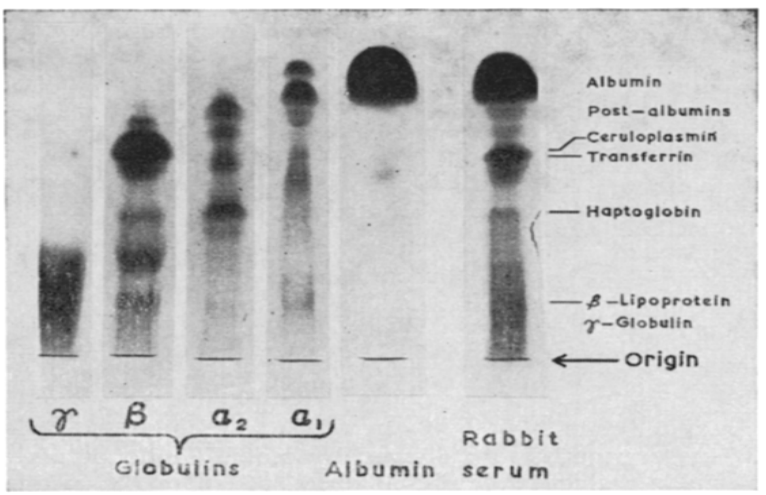

Fig. 4. Stained starch gel electrophoretic patterns of fractions obtained by PVK zone electrophoresis of rabbit serum in Tris buffer.

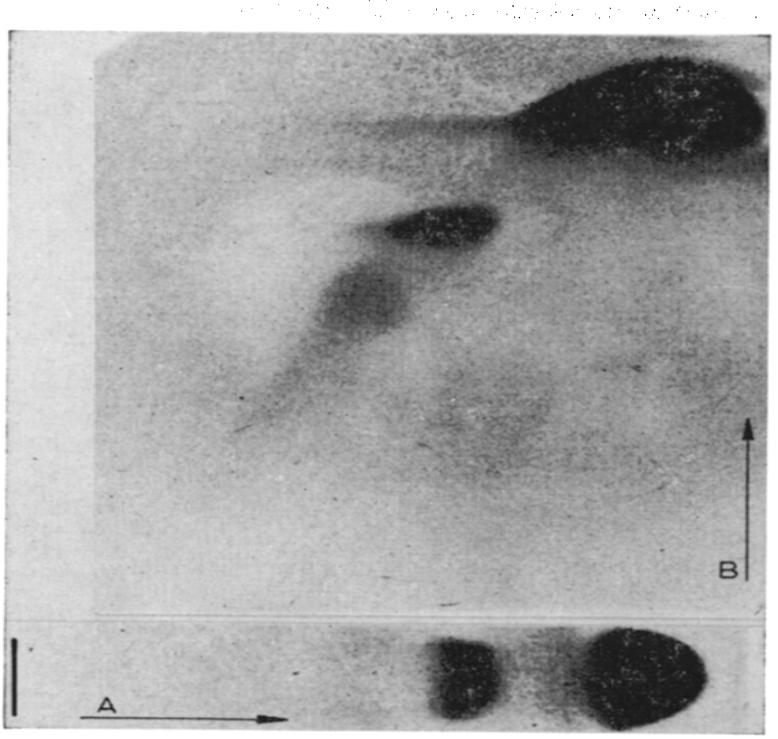

Fig. 5. (A) First and (B) second-dimension starch-gel electrophoresis of albumin obtained by PVK zone electrophoresis in barbital buffer.

In order to isolate single proteins the whole fraction was labelled with ^{131}I and the components were located by autoradiography on the starch gel slab.

Selected segments were then transferred to a PVK bed for elution of the proteins. ^{131}I-Proteins were recovered almost quantitatively in the PVK, and after concentration the samples were re-examined on starch gel (Fig. 7). Immunoelectrophoresis of this transferrin gave only one precipitin arc.

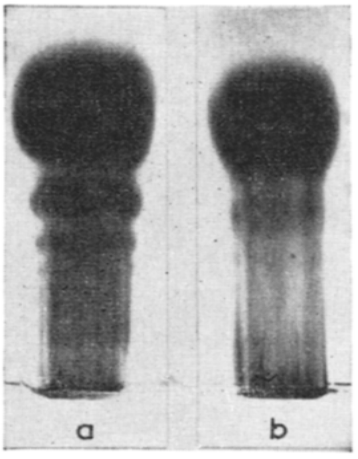

Fig. 6. Effect of 0.06 *M* mercaptoethanol followed by 0.02 *M* iodoacetamide on PVK albumin. Electrophoresis in starch gel in the presence of 8 *M* urea. (a) Untreated albumin; (b) albumin after treatment.

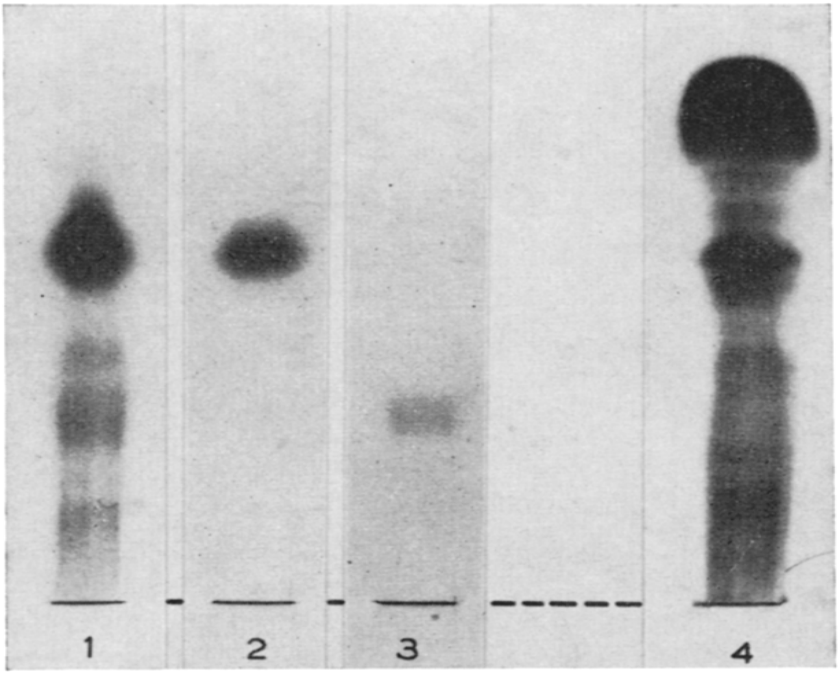

Fig. 7. Stained starch gel electrophoretic patterns of (1) β-globulins as obtained by PVK zone electrophoresis; (2) transferrin component from (1); (3) unidentified middle component from (1); (4) rabbit serum.

DISCUSSION

It appears that PVK zone electrophoresis gives only albumin and γ-globulin in "homogeneous" form, both comprising a variety of molecules having slightly different mobilities in starch gel (microheterogeneity according to COLVIN *et al.*[26]).

α_1-, α_2- and β-globulin PVK fractions appear to be very complex. Thus, seven radioactive fractions were found when ^{131}I-β-globulins were separated on starch gel.

It is obvious that, for the time being, it is only practical to attempt the isolation of major fractions with PVK. The procedure proposed in this study may provide a practical as well as an accurate method of doing this. Plasma (loading up to 0.9–1.0 g total proteins) is first separated to get γ-globulin and albumin. The α_1-, α_2- and β-globulins require further separation by starch gel electrophoresis and protein recovery by elution in a starch gel–PVK system.

Purification of the β-globulins was repeated twice with apparently satisfactory results in terms of transferrin yield. It is felt that attempts to isolate minor β-components are not worth while unless about 80 mg of crude β-globulins are available.

Albumin also was completely freed from its minor components by this technique (Fig. 3), the recovery being 80–90 %.

The alternative of applying ion-exchange chromatography appears laborious and possibly less effective. In fact, rabbit albumin prepared by using CM-cellulose[14–16] appeared, on starch gel, to be more complex than albumin obtained by PVK zone electrophoresis.

The localisation of protein bands in PVK has proved to be easy and reliable. The use of ultraviolet light is effective in following the progress of electrophoresis. Autoradiography has also been found useful for locating proteins on the PVK block or on the starch gel slab. The use of ^{131}I-proteins is permissible even if ^{14}C-proteins have to be measured by scintillation counting. Overall recoveries of proteins were between 60 and 70 %. The tendency of protein boundaries to spread during zone electrophoresis is reduced when the precautions outlined here are observed, but it is always advisable to select for elution only the centre of the band in order to obtain a homogeneous albumin. In the experimental conditions described albumin was always well separated from α_1-globulins.

A comment is needed on the presence of more than one component when albumin prepared by PVK zone electrophoresis is tested on starch gel. The heterogeneity of the albumin has been discussed many times (HOCH-LIGETI AND HOCH[27], SAIFER AND COREY[28–29], LARSON AND FEINBERG[30], TISELIUS *et al.*[31], SOBER *et al.*[15], KELLER AND BLOCK[32], SAIFER AND ROBIN[33]). The formation of albumin dimer was reported by STRAESSLE[34], and KING *et al.*[35] separated the dimer from the monomer. POULIK AND SMITHIES[36] and SAIFER *et al.*[37] stated that when human serum was first resolved by paper electrophoresis and then by starch gel electrophoresis the albumin did not resolve into more than one component. However, in a recent paper POULIK *et al.*[38] have described the presence of an albumin-like component in human sera that was immunologically related to albumin and disappeared in the presence of reducing agent.

The minor electrophoretic components seen in albumin prepared by PVK zone electrophoresis disappeared after treatment with S–S bond splitting reagents. PVK albumin gave only a single precipitation arc in the albumin area. This behaviour suggests the presence of albumin polymers in rabbit plasma.

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SUMMARY

Rabbit plasma proteins (0.9–1.0 g) were separated by zone electrophoresis in Pevikon C-870, using different buffers. Viewing by ultraviolet light and autoradiography were found to be helpful in locating the fractions, and starch gel electrophoresis and immunoelectrophoresis were used to define their composition in greater detail; only albumin and γ-globulin proved to be homogeneous. Homogeneous transferrin was obtained in useful quantity by further separating the β-globulin fraction by starch gel electrophoresis.

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