STARCH–PEVIKON C-870 GEL AS A SUPPORTING MEDIUM IN ZONE ELECTROPHORESIS

VELIO BOCCI

Istituto di Fisiologia Generale della Università di Siena, Siena (Italy)

(Received December 14th, 1962)

INTRODUCTION

Direct procedures for elution of proteins from starch gel have been discussed by SMITHIES[1]. More successful techniques allowing almost quantitative recoveries have been described[2–5], but appear to be rather time-consuming. Attempts to recover faster moving components by means of a fraction collector and the combined use of an electrostatic field and a buffer flow crossing a vertical gel have not given, so far, any significant results.

A preliminary account concerning the use of a gel formed by hydrolysed starch and a powdered co-polymer of polyvinyl chloride and polyvinyl acetate, "Pevikon C-870", has been reported[6]. The combination of starch with a non-swelling medium produced a gel with a high resolving power for protein mixtures and little tendency to adsorb proteins.

The present paper gives conclusive evidence that by means of starch–PVK* gel electrophoresis, serum proteins as well as soluble liver and brain proteins can be satisfactorily resolved; furthermore, data referring to recoveries of protein from starch–PVK gel and starch gel are presented and the procedure for elution of proteins from starch–PVK gel is described.

MATERIALS AND METHODS

Rabbit and human serum proteins were used throughout the experiments. Rabbit haemoglobin was prepared according to NYMAN[7].

Brain and liver of a heparinized rabbit (5 mg heparin/kg body-weight), after intravascular perfusion of warm saline, were cut up into small slices and homogenized with an equal amount of distilled water; the homogenates were frozen-thawed 7 times prior to centrifugation for 50 min at 34,000 × g in a Lourdes Model AX centrifuge. The whole procedure was carried out at 2–6°.

Protein solutions were labelled with carrier-free ^{131}I (The Radiochemical Centre) without oxidation, according to MCFARLANE[8]. Free iodide was removed by passing the solutions through anion exchange columns of De-Acidite (The Permutit Co. Ltd., London, W. 4) and then by dialysis against the buffer used for electrophoresis.

* Abbreviations: PVK = Pevikon C-870; TCA = trichloroacetic acid; Tris = tris-(hydroxymethyl)-amino-methane.

The small precipitates formed during dialysis were removed by centrifugation. ^{131}I-TCA soluble radioactivity as a percentage of the original radioactivity (after precipitation with TCA at a final concentration of 10 % in the presence of NaI and carrier rabbit serum proteins) was: brain, 18.1; liver, 5.3; haemoglobin, 6.1; serum, 2.4.

Starch–PVK gel was prepared as follows: starch (Connaught Medical Research Laboratories), 10.5 g, and PVK (Stockholms Superfosfat Fabriks Aktiebolag, Stockholm), 126.0 g, were thoroughly mixed with 100 ml Tris–citric acid buffer (POULIK[9]) and the suspension was heated as indicated by SMITHIES[10]. The mixture became almost solid at 50–60° but further heating and vigorous swirling of the flask produced a solution of suitable viscosity. In order to produce a homogeneous solution heating was prolonged for about 10 min to a point just short of boiling.

Starch gel was prepared at a concentration of 10.5 g of starch per 100 ml buffer.

A tray of 30.5 × 21.0 × 1.0 cm was usually used in horizontal position with a discontinuous buffer system according to POULIK[9]. The origin was at 7.5 cm from the cathodal end of the gel slab and the protein solutions were taken up in Ford's A 4 filter paper strips (about 0.065 ml per 1 cm^2 of filter paper). The solutions had the following protein concentration (mg/ml): brain, 21; liver, 45; haemoglobin, 33; serum, 72.

After the insertion of the sample a proper contact was insured by pushing the cathodal end of the gel towards the origin with a perspex strip and filling the space with wet filter paper. A voltage gradient of 6 V/cm was employed until the borate front reached the origin and then it was increased up to 19 V/cm (current of 80–90 mA); electrophoresis was considered complete when the borate front was about 12.5 cm from the origin towards the anode. Increasing the voltage gradient resulted in sharper and better protein separation and it shortened the run down to about 6.5 h. The electrophoretic run was performed at room temperature but the table, the polythene cushion resting on top of the gel and the borate bridge buffer[9] (3.7 l for each bicompartmented vessel) were all cooled with running tap water.

Protein recoveries were measured as follows: the gel slab was sliced longitudinally and holes were made at 1.0 cm intervals using a needle. With the help of the needle holes protein bands were localized after staining and washing (SMITHIES[10]) the top slice of gel or, if the proteins were highly radioactive, by carrying out autoradiography directly on the lower slice of gel (Kodirex, X-Ray film). Selected segments (2.0 × 1.0 × 0.4 – 0.5 cm) of the lower slice were put in glass counting tubes and saline was added up to a volume of 3 ml. Some of the samples were kept at + 3° for different lengths of time, some were frozen at — 40° and thawed three times.

When the samples had been counted for total radioactivity in a well-type scintillator, proteins were eluted by squeezing the segments in a metal-glass syringe, a filter paper disc preventing the gel mass escaping. After centrifugation the clear supernatant was brought up to 3 ml and counted for calculating the percentage of recovered radioactivity. The standard deviation of counts recorded in this study did not exceed ± 1 %.

When proteins were to be eluted from large segments of gel the final procedure was as follows: saline was added to the starch–PVK gel so that it could be easily smashed with a spatula to a slurry consistency. The sample was counted for total radioactivity and, after centrifugation, the clear supernatant, having been brought to the original volume, was counted for measuring the percentage of recovered radioactivity. The

starch–PVK sediment could be washed in order to increase recoveries. For comparative purposes starch gel was treated in the same way except that the gel had to be smashed by an MSE homogeniser.

Proteins were concentrated by dialysis (Visking tubing 8/32 in.) under reduced pressure. The precipitated starch at the bottom of the bag, was eliminated by centrifugation.

In order to attempt the separation of soluble starch from proteins, a column (3.0 × 128.0 cm) with Sephadex G-100 (Pharmacia, Uppsala) was used for gel filtration[11] of eluates obtained from starch–PVK gel after electrophoretic separation of human ^{131}I-serum. A glass paper disc (Whatman GF/B) was placed on the top surface of the gel bed. Elution was carried out with 0.005 *M* NaCl in 0.005 *M* potassium phosphate buffer, pH 7.1. Elution rate: 26 ml/h.

Proteins were estimated from 280 mμ, 407 mμ and 540 mμ (GORNALL *et al.*[12]) absorption in a Unicam SP.500 spectrophotometer. A crystallized sample of bovine serum albumin (Sigma Co.) was used as a standard.

RESULTS

The starch–PVK gel was white and the mechanical strength slightly inferior to that of starch gel. Using a discontinuous buffer system starch–PVK gel shrank very little as the borate front progressed, thus causing only occasionally an imperfect contact at the origin.

Only 1.5 % of total ^{131}I-labelled proteins remained at the origin. In comparison with starch gel, proteins enter starch–PVK gel more slowly, and the albumin mobility is about 1/10 lower. Also with starch–PVK gel a sharpening of protein bands was observed when they were reached by the borate front.

Slicing of the gel presented no difficulties. Excess dye was more quickly washed out of starch–PVK gel due to the lack of adsorption of dye by the PVK. A useful property of starch–PVK gel is the absence of shrinkage after washing, thus making the localization of protein bands on the unstained gel easier and more precise.

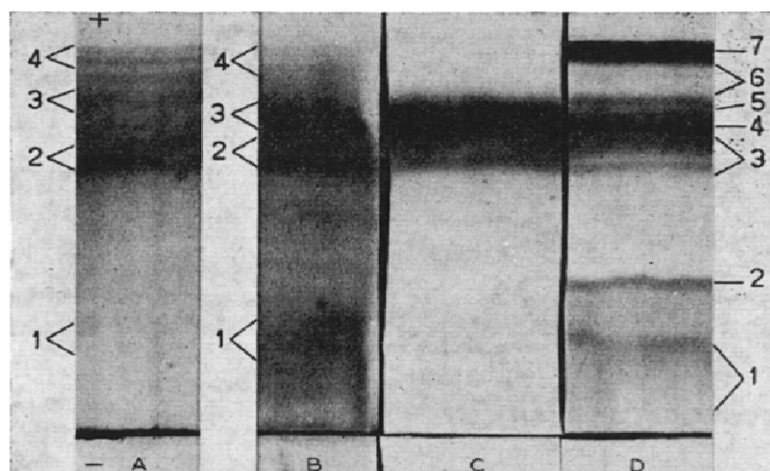

Fig. 1. Patterns of rabbit brain (A) and liver (B) soluble proteins, of haemoglobin (C) and serum proteins (D) obtained by starch–PVK gel electrophoresis. Nos. 1–4 (A,B) refer to the protein bands selected for elution (Table I). Numbers of (D) correspond to: (1) β-lipoprotein in γ-globulin region; (2) S α_2-globulin; (3) haptoglobins; (4) transferrin; (5) ceruloplasmin; (6) post-albumins; (7) albumin.

When using starch–PVK gel human serum protein recoveries increased up to 39 % and therefore it was thought worth while to investigate the degree of separation of tissue soluble proteins and to measure their recoveries.

Protein patterns as obtained by starch–PVK gel electrophoresis are shown in Fig. 1.

No attempts were made to identify the enzymatic activity of brain and liver soluble proteins and they were numbered arbitrarily from cathode to anode and recoveries of corresponding segments were measured from starch and starch–PVK gels. The results are listed in Table I.

TABLE I

PROTEIN RECOVERIES (AS PERCENTAGES) AFTER STARCH–PVK GEL AND STARCH GEL ELECTROPHORETIC SEPARATION OF RABBIT BRAIN AND LIVER ^{131}I-PROTEINS

Gel segments were frozen and thawed three times. Percentages of ^{131}I-TCA soluble radioactivity were estimated on the eluates and are given in parentheses.

*Protein fractions**	*Protein recovery*			
	Brain soluble ^{131}I-proteins		*Liver soluble ^{131}I-proteins*	
	Starch	*Starch–PVK*	*Starch*	*Starch–PVK*
1	11 (5.6)	40 (4.8)	17 (4.8)	39 (3.6)
2	33 (4.8)	61 (3.7)	31 (3.4)	52 (3.1)
3	55 (5.0)	66 (4.0)	50 (4.9)	68 (3.2)
4	75 (19.3)	76 (16.7)	78 (6.7)	81 (5.1)

* The numbers of the protein fractions refer to those in Fig. 1.

Protein recoveries were higher from starch–PVK gel and percentages of ^{131}I-TCA soluble radioactivity were lower than in starch gel.

Freezing may alter proteins and increase ^{131}I-TCA soluble radioactivity. In order to increase recoveries a very mild condition such as storing gel segments in saline at + 3° was tried.

The results showed (Table II) that higher protein recoveries were obtained when proteins diffused from gel into saline rather than freezing and thawing gels in order to disrupt their structure. Freezing of proteins resulted in consistently higher values of ^{131}I-TCA soluble radioactivity.

Protein recoveries, expressed as percentages of total radioactivity, increased considerably for both gels in the first 15 h and then progressively less until they reached the maximum after 90 h. Recoveries from starch gel segments left in saline as long as 90 h were, except for γ-globulin, only slightly lower when compared with corresponding starch–PVK gel segments, the eluates of the latter having, however, lower ^{131}I-TCA soluble radioactivity. Nevertheless from these results starch–PVK gel did not show, as far as protein recoveries were concerned, a striking advantage over starch gel.

Proteins diffused spontaneously from the gel because the saline became increasingly coloured when gel segments contained coloured proteins. From separate radioactivity measurements of saline and gel segments it appeared that squeezing the gel increased recoveries by no more than 10 %. Apparently free ^{131}I diffused from gel segments more rapidly than ^{131}I-proteins: ^{131}I-TCA soluble radioactivity appeared higher in the first 15 h than after 45 h.

TABLE II

PROTEIN RECOVERIES (AS PERCENTAGES) AFTER STARCH–PVK GEL AND STARCH GEL ELECTROPHORETIC SEPARATION OF RABBIT SERUM ^{131}I-PROTEINS AND OF RABBIT ^{131}I-HAEMOGLOBIN

Columns (A), (B), (C) and (D) refer to gel segments left in saline at + 3° for 2, 15, 45 and 90 h respectively. Columns (E) refer to gel segments kept frozen in saline at —40° and thawed three times during 90 h. All segments came from the same gel slabs. Percentages of ^{131}I-TCA soluble radioactivity were estimated on the eluates and are given in parentheses. Percentages of calculated ^{131}I-radioactivity protein bound are underlined.

Protein fractions	Protein recovery									
	A		B		C		D		E	
	Starch gel	*Starch–PVK gel*	*Starch gel*	*Starch–PVK gel*	*Starch gel*	*Starch–PVK gel*	*Starch gel*	*Starch–PVK gel*	*Starch gel*	*Starch–PVK gel*
γ-Globulin	9.0	15.6	14.0	35.0	29.4 (12.0) 25.9	46.6 (8.2) 42.8	34.3 (14.4) 29.4	57.8 (8.1) 53.1	14.6 (24.5) 11.0	44.5 (7.9) 41.0
Sα_2-Globulin*	8.4	25.7	26.1 (5.5) 24.7	42.0 (4.6) 40.1	44.8 (3.9) 43.1	63.0 (3.6) 60.7	55.4 (3.9) 53.2	69.1 (4.0) 66.3	11.7 (15.4) 9.9	44.8 (5.8) 42.2
Transferrin	17.1 (4.3) 16.4	29.3 (2.4) 28.6	40.4 (3.3) 39.1	59.4 (2.1) 58.2	60.4 (2.5) 58.9	74.3 (1.7) 73.0	73.5 (2.6) 71.6	85.5 (1.9) 83.9	43.4 (6.7) 40.5	79.6 (2.4) 77.7
Albumin	—	—	—	—	—	—	91.1 (2.7) 88.6	93.2 (2.4) 91.0	89.4 (2.9) 86.8	92.5 (2.4) 90.3
Haemoglobin	19.2 (19.4) 15.5	26.1 (7.2) 24.2	45.9 (11.3) 40.7	59.6 (7.0) 55.4	65.1 (13.5) 56.3	70.5 (5.3) 66.8	75.8 (10.9) 67.5	77.4 (5.8) 72.9	66.4 (15.1) 56.4	61.2 (7.5) 56.6

* This protein has been compared[13] to the human "slow" α_2-globulin.

TABLE III

PROTEIN RECOVERIES (AS PERCENTAGES) AFTER STARCH–PVK GEL AND STARCH GEL ELECTROPHORETIC SEPARATION OF HUMAN SERUM ^{131}I-PROTEINS

All proteins were eluted excluding only about 1 mm of gel at the origin. To both gels, saline equal to their volumes was added three times. Percentages of ^{131}I-TCA soluble radioactivity were estimated on the eluates and are given in parentheses.

Saline washings	*Protein recovery*	
	Starch gel	*Starch–PVK gel*
First	35 (2.6)	59 (2.5)
Second	18 (2.4)	24 (2.4)
Third	7	11 (2.5)

Finally the usefulness of using starch–PVK gel was mainly due to high protein recoveries being obtained with a very simple procedure (see Methods). The results are listed in Tables III and IV.

Starch–PVK gel allowed an overall increase of 34 % in serum protein recoveries. This increase was quite consistent for all proteins except albumin. ^{131}I-Albumin which retained about 65 % of total ^{131}I-serum radioactivity was always well recovered from both gels.

Protein fractions recovered from starch–PVK gel retained their original electrophoretic mobilities; moreover, judging from the absence of radioactive proteins at the origin, very little denaturation seemed to have occurred (Fig. 2). By autoradiographic estimation neither trailing nor tailing effects were observed after starch–PVK gel electrophoresis of ^{131}I-labelled protein fractions.

TABLE IV

PROTEIN RECOVERIES (AS PERCENTAGES) AFTER STARCH–PVK GEL AND STARCH GEL ELECTROPHORETIC SEPARATION OF HUMAN SERUM ^{131}I-PROTEINS

Proteins were eluted once, adding volumes of saline to one volume of gel as specified. Percentages of ^{131}I-TCA soluble radioactivity were estimated on the eluates and are given in parentheses

Protein fractions	*Gel: saline ratio*	*Protein recovery*		*Increase in recovery*
		Starch gel	*Starch–PVK gel*	
γ-Globulin	1:3	27 (12.3)	67 (6.8)	40
Sα_2-Globulin	1:3	44 (4.2)	63 (3.5)	19
Haptoglobins	1:3	50 (9.8)	75 (4.8)	25
Transferrin	1:4	61 (3.8)	76 (1.6)	15
Post-albumins	1:2	38	75	37
Albumin	1:5	73 (2.8)	77 (2.4)	4

Contents of soluble starch and protein contaminants from a blank experiment were measured and the results of a typical experiment are reported in Table V.

The amount of soluble starch eluted from a starch–PVK gel slab was lower than that eluted from a starch gel slab of identical volume. However, because starch–PVK gel contained 12 times as much PVK as starch the percentage of eluted starch was comparatively higher for starch–PVK gel. The amount of material reacting as protein by the method of GORNALL *et al.*[12] in eluates also obtained from gel slabs of identical

volume, were rather similar or equal, and the ^{131}I-protein bound radioactivity/protein amount (in mg) ratios were also equal or very similar for rabbit haemoglobin and albumin eluted from both gels.

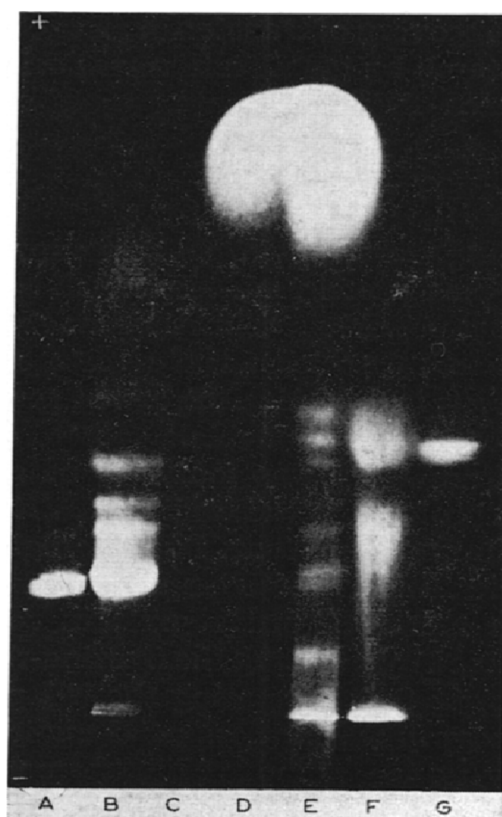

Fig. 2. Starch gel autoradiographs of ^{131}I-labelled human serum proteins: (A) Sα_2-globulin; (B) α_2-globulins obtained after PVK block electrophoresis[15] of serum proteins; (C) ceruloplasmin; (D) albumin; (E) whole serum; (F) β-globulins obtained as B; (G) transferrin. (A), (C) and (G) were obtained after starch–PVK electrophoresis of (B) and (F). (D) was obtained after starch–PVK gel electrophoresis of whole serum.

Gel filtration of protein eluates obtained from starch–PVK gel did not produce any separation of soluble starch from proteins with molecular weights higher than about 100,000: most of the soluble starch, detectable by addition of iodine to the

TABLE V

CONTENTS OF SOLUBLE STARCH AND PROTEIN (BLANK) IN STARCH GEL AND STARCH–PVK GEL SLABS AFTER ELECTROPHORESIS

Saline was added in equal volume twice and the eluates were pooled.

Samples	*Volume (ml)*	*Wet weight (g)*	*Dry weight (g)*	*Eluted soluble starch*		*Protein reacting material (mg)*
				as total amount (mg)	*as % of dry weight*	
Starch gel	21	23.0	2.17	210	9.7	27.5
Starch–PVK gel	21	25.6	15.60 (1.20)+	170	14.2	24.9

* Refers to the amount of starch present in starch–PVK gel.

samples, appeared between 250–259 ml of effluent together with a first protein peak namely γ-globulin and β-lipoprotein, whereas a second protein peak due either to transferrin or albumin (301–315 ml of effluent) still contained traces of undialysable starch. Recoveries of ^{131}I-proteins from a Sephadex G-100 column ranged between 87–95 %. However, the final recovery of transferrin after concentration by dialysis and elimination of most of the starch by gel filtration was only 51 % in comparison with 83 % as measured in the starch–PVK gel eluate.

DISCUSSION

Zone electrophoresis using starch–PVK gel as a supporting medium has proved useful for fractionating complex protein mixtures such as serum proteins and tissue soluble proteins. The resolving power of the method was improved by using a discontinuous buffer system in horizontal position and a voltage gradient of about 20 V/cm. Starch–PVK gel electrophoresis in a vertical direction could be troublesome if a tray with a removable glass plate was not available.

Recovery of proteins from PVK was found to be almost quantitative[14,15]: in order to reduce adsorption of proteins by the medium, PVK was mixed with hydrolysed starch in the hope of obtaining a gel that, besides high resolving power, allowed satisfactory protein recoveries. In fact with a very simple procedure for eluting proteins the overall recoveries increased up to 34 % in comparison with starch gel. Further advantage was obtained by avoiding the freezing-thawing procedure: ^{131}I-TCA soluble radioactivity was lower thus increasing the ^{131}I-protein bound radioactivity recoveries. When a high recovery was not essential undiluted protein fractions were obtained by just adding a few drops of saline to facilitate melting of the starch–PVK gel.

Minor advantages were: the possibility of readily detecting coloured proteins on a white background, the quick washing out of excess dye and the practical absence of shrinkage of the gel. Starch gel shrank about 14 % and this fact made localisation of proteins uncertain on the unstained gel.

Radioactive protein fractions did not show any trailing in starch–PVK gel autoradiographs and proteins travelled at the same rate throughout the thickness of the gel thus reducing the possibility of contamination and increasing protein recoveries. Although electrophoresis was performed at room temperature with a relatively high current, a vertical deformation of protein bands was avoided by applying a polythene cushion on top of the gel, which prevented evaporation and permitted cooling of the upper surface of the gel with running tap water.

Using the gel slab described, up to 120 mg of serum proteins were satisfactorily separated. The usefulness of the method was demonstrated in one experiment, when by using a slab of 31.0 × 1.2 × 24.0 cm as much as 360 mg of human serum proteins were separated after being taken up in two filter paper strips.

However, starch–PVK gel was not considered as a medium for preliminary protein fractionation but rather as a supporting gel suitable for final purification of simple protein mixtures. Resolution of protein components by either starch gel or starch–PVK gel one-dimensional electrophoresis was not feasible because of an overlapping of protein fractions. Albumin prepared in this way formed a single zone only when re-run in starch gel but it showed globulin contaminants when examined by cellulose acetate electrophoresis according to KOHN[16].

Two electrophoretic separations of rabbit serum carried out under different conditions were able to yield pure protein fractions, as was previously described[15]. Starch–PVK gel electrophoresis therefore can be employed as a final purification step, coupling the advantages of good resolving power with low adsorption for proteins.

A disadvantage of starch–PVK gel (shared by starch gel) was the contamination of proteins with rather large amounts of soluble starch that made any measurement of the carbohydrate content of proteins unreliable. Gel filtration of protein solutions did not separate soluble starch completely from albumin or transferrin as was, for example, possible by chromatography according to DE PAILLERETS *et al.*[17] or by electrophoresis[5].

ACKNOWLEDGEMENT

This work was supported by a grant from the Consiglio Nazionale delle Ricerche.

SUMMARY

A gel composed of hydrolysed starch and powdered co-polymer of polyvinyl chloride and polyvinyl acetate (Pevikon C-870) is proposed as a supporting medium for separating serum proteins and soluble tissue proteins by electrophoresis. The procedure for eluting proteins from starch–PVK gel is very simple and recoveries are much higher than from starch gel. ^{131}I-trichloroacetic acid soluble radioactivity is consistently lower when proteins are recovered from starch–PVK gel. The presence of soluble starch does not affect the estimation of the specific activity of ^{131}I-labelled proteins.

REFERENCES

1 O. SMITHIES, *Advan. Protein Chem.*, 14 (1959) 81.
2 J. MORETTI, G. BOUSSIER AND M. F. JAYLE, *Bull. Soc. Chim. Biol.*, 40 (1958) 59.
3 P. JARRIGE AND G. LAFOSCADE, *Bull. Soc. Chim. Biol.*, 41 (1959) 1197.
4 A. H. GORDON, *Biochim. Biophys. Acta*, 42 (1960) 23.
5 V. BOCCI, *J. Chromatog.*, 6 (1961) 357.
6 V. BOCCI, *Nature*, 197 (1963) 491.
7 M. NYMAN, *Scand. J. Clin. Lab. Invest.*, Suppl. 39 (1959) 43.
8 A. S. MCFARLANE, *Nature*, 182 (1958) 53.
9 M. D. POULIK, *Nature*, 180 (1957) 1477.
10 O. SMITHIES, *Biochem. J.*, 61 (1955) 629.
11 P. FLODIN, *Dextran Gels and Their Applications in Gel Filtration*, Pharmacia, Uppsala, 1962.
12 A. G. GORNALL, C. J. BARDAWILL AND M. M. DAVID, *J. Biol. Chem.*, 177 (1949) 751.
13 G. RIOU, C. PAOLETTI AND R. TRUHAUT, *Bull. Soc. Chim. Biol.*, 44 (1962) 149.
14 H. J. MÜLLER-EBERHARD, *Scand. J. Clin. Lab. Invest.*, 12 (1960) 33.
15 V. BOCCI, *J. Chromatog.*, 8 (1962) 218.
16 J. KOHN, *Clin. Chim. Acta*, 2 (1957) 297.
17 C. DE PAILLERETS, J. MORETTI AND M. F. JAYLE, *Bull. Soc. Chim. Biol.*, 41 (1959) 1285.