STUDIES ON THE CHROMATOGRAPHY OF HUMAN SERUM PROTEINS ON DIETHYLAMINOETHYL(DEAE)-CELLULOSE

II. THE CHROMATOGRAPHIC CHARACTERISTICS OF PURIFIED HUMAN SERUM PROTEINS

K. JAMES AND D. R. STANWORTH

Department of Experimental Pathology, The Medical School, University of Birmingham (Great Britain)

(Received December 2nd, 1963)

INTRODUCTION

Although, as already mentioned[1], DEAE-cellulose chromatography has been used extensively in the fractionation of serum proteins, there have been no previous reports of the investigations of well characterized individual proteins on this exchangers.

In the work to be described the chromatographic properties of several purified serum proteins have been studied. The group of proteins studied comprised macromolecules with a wide range of molecular size and charge density.

The effect of protein–protein interaction has also been determined by the chromatography of artificial protein mixtures, some of which included isotopically labelled proteins.

Studies of this type are essential if the resolution of serum protein mixtures affected by DEAE-cellulose chromatography is to be improved and if new selective isolation procedures are to be developed.

METHODS

Purified proteins

The human serum proteins used in these investigations are listed in Table I, together with their mode of preparation. The 7 S-γ-globulin, β-lipoprotein and α_2-macroglobulin preparations were shown to be free of other serum proteins by ultracentrifugal analysis and immunoelectrophoretic analysis employing a rabbit antiserum to whole human serum. By similar techniques the siderophilin preparations were found to contain small amounts of contaminating 7 S-γ-globulin (less than 5 %) and the albumin, a β-globulin impurity (2–5 %). In the case of the Lister albumin preparation the β-globulin contaminant was siderophilin.

Isotopic labelling of proteins

Various protein preparations were labelled with ^{131}I by the iodine monochloride technique of McFARLANE[6], as modified by DAVIES *et al.*[7], whilst siderophilin was labelled with ^{59}Fe by an exchange procedure (VEAL AND VETTER[8]). The activity of

TABLE I

METHODS OF PREPARATION OF THE PROTEINS INVESTIGATED

Protein	*Sample numbers*	*Method of separation*
7 S-γ-Globulin	1	DEAE-cellulose column chromatographic separation from normal human serum
	2	DEAE-cellulose batch chromatographic separation from outdated acid citrate dextrose plasma (STANWORTH[2])
Siderophilin	1 and 3	DEAE-cellulose column chromatographic separation of F IV–4 obtained by the low temperature ethanol procedure of COHN *et al.*[3]
	2	DEAE-cellulose chromatographic separation of outdated acid citrate dextrose plasma followed by further DEAE-cellulose chromatography of the siderophilin-rich fraction
β-Lipoprotein	1	Subfractionation of Fractions II and III, obtained by COHN method 6[3], by method 9 (ONCLEY *et al.*[4]) to give fraction III-O; zone centrifugation of this fraction in saline density gradient
α_2-Macroglobulin	1	Zone centrifugation in sucrose density gradient of the pellet obtained in the above centrifugation procedure
Albumin	1	Fraction V obtained by COHN method 6[3]
	2	Commercial preparation (LISTER) obtained by the ether fractionation procedure of KEKWICK AND MACKAY[5]

labelled fractions was determined by counting measured aliquots in a thallium activated, well shaped, sodium iodide crystal.

Column chromatographic procedure

With one exception (discussed later) all chromatographic separations were carried out in columns containing 2 g of DEAE-cellulose exchanger prepared in the laboratory from wood cellulose (Solka Floc Grade BW 100) according to the method of PETERSON AND SOBER[9] (column dimensions 16.0 × 1 cm). This material had a degree of substitution of 0.87 mequiv./g. Elution was effected by a stepwise procedure using the following series of solvents:

1. Phosphate buffer: pH 7.6, 0.01 *M*.
2. Phosphate buffer: pH 6.3, 0.02 *M*.
3. 0.05 *M* NaH_2PO_4 solution.

Other practical details are discussed in the previous paper[1].

RESULTS

(a) Studies of single proteins

The amounts of protein eluted with the various solvents are recorded in Table II in which the mean recoveries and the standard deviation of the mean are given. Statistical analysis revealed that the total recoveries of 7 S-γ-globulin and albumin were significantly greater than those observed with total serum protein, whilst the recovery of siderophilin was comparable. On the other hand, the recoveries of β-lipoprotein and α_2-macroglobulin were significantly less than that shown by total serum protein.

TABLE II

THE AMOUNTS OF THE VARIOUS SERUM PROTEINS CHROMATOGRAPHED AND THEIR RECOVERIES

Mean recoveries expressed together with their standard deviations ($6\bar{x}$).

Sample	*Number of experiments*	*Amount protein applied (mg)*	*Protein recovery (percentage of total applied)*			
			Phosphate buffer		*0.05 M NaH_2PO_4*	*Total*
			pH 7.6, 0.01 M	*pH 6.3, 0.02 M*		
Normal human serum	4	365	8.2 ± 2.3	7.5 ± 1.1	57.4 ± 6.8	73.0 ± 8.8
7S-γ-globulin	4	20.5 ± 4.2	72.4 ± 4.6	11.9 ± 4.4	6.1 ± 2.6	90.5 ± 3.9
Siderophilin	5	119.4 ± 25.6	52.7 ± 8.0	10.1 ± 3.7	2.9 ± 0.8	65.7 ± 7.3
β-Lipoprotein	2	13.5	0.65 ± 0.65	7.2 ± 3.2	15.4 ± 3.7	23.1 ± 6.4
α_2-Macroglobulin	4	70.1 ± 4.6	2.7 ± 0.9	0.9 ± 0.2	29.4 ± 3.0	33.4 ± 3.1
Albumin	6	157.0 ± 47.8	0.8 ± 0.2	1.1 ± 0.2	82.1 ± 1.3	84.1 ± 1.2
7S-Globulin and siderophilin	3	51.7 ± 15.3	61.0 ± 6.7	13.1 ± 3.9	1.3 ± 0.4	75.2 ± 5.0
7S-Globulin and albumin	2	125 ± 15.0	32.8 ± 4.6	2.4 ± 0.2	52.9 ± 4.9	88.2 ± 0.3
α_2-Macroglobulin and albumin	1	114	0.3	0.8	37.0	38.1
7S-Globulin, siderophilin, α_2-macroglobulin and albumin	1	168	18.9	15.7	46.9	81.5

As was to be expected most of the 7S-γ-globulin failed to bind to the exchanger and so was recovered in the initial solvent front. Nevertheless, this protein exhibited the "trailing" effect also observed by other investigators[10,11].

The elution characteristics of isolated siderophilin were found to differ markedly from those exhibited by this protein during the DEAE-cellulose chromatography of whole serum[12]. For instance, the major part of the isolated siderophilin was eluted by the first solvent (0.01 *M* phosphate, pH 7.6) whereas in separation of whole serum it was completely eluted by the second solvent (0.02 *M* phosphate, pH 6.3). This effect does not appear to have been observed by TOMBS *et al.*[13], in their studies on the chromatographic behaviour of electrophoretically prepared β-globulin fraction.

Recoveries of β-lipoprotein were extremely low (23.1 % ± 6.4), the eluted material being dispersed throughout the greater part of the chromatogram. Similarly the recovery of α_2-macroglobulin was disappointingly low (33.4 % ± 3.1). In contrast to the β-lipoprotein, however, almost all of this protein was recovered with one solvent (the 0.05 *M* NaH_2PO_4 solution). It was eluted ahead of the normal 19S-γ-globulin position[11], and also prior to the albumin (see Fig. 1).

As mentioned earlier, the total recoveries of albumin were constantly high (84.1 % ± 1.2). Again the major portion of this protein (82.1 % ± 1.3) was recovered with the final solvent (0.05 *M* NaH_2PO_4 solution). The chromatographic patterns obtained by duplicate analysis of the same albumin sample showed considerable variability, however. Compare for instance patterns A and B in Fig. 2 which were obtained by parallel analyses on the same albumin preparation (a COHN Fraction V). Comparison with patterns (E and F) given by larger amounts of a different albumin preparation (LISTER) revealed even greater variation. It should be mentioned, however, that the leading minor peaks in these patterns can be attributed to contaminating siderophilin.

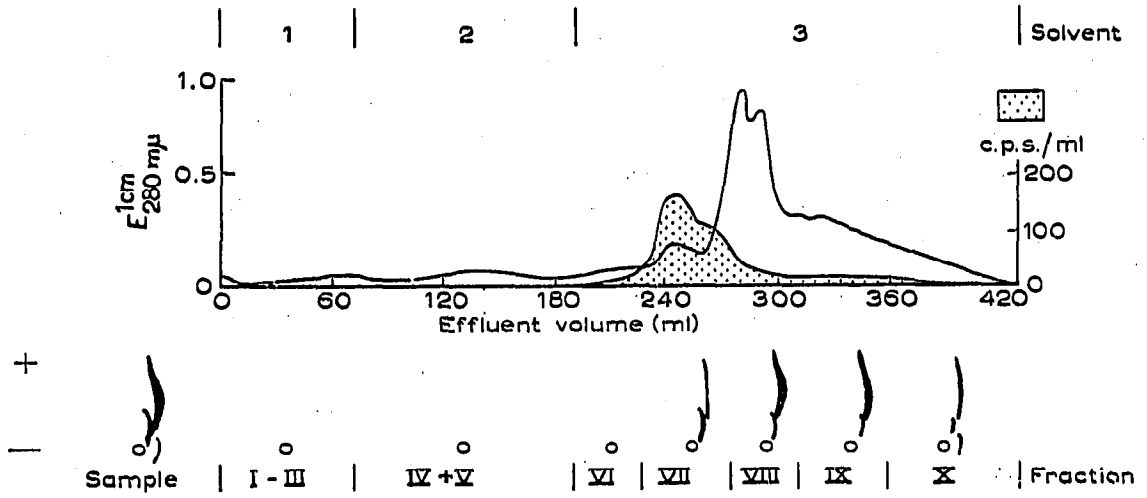

Fig. 1. Chromatography of a mixture of ^{131}I labelled α_2-macroglobulin (12.6 mg) and COHN F V albumin (101.4 mg of preparation 1) on DEAE-cellulose exchanger W. For chromatographic details see text.

In general, the recoveries of the various proteins studied were found to be independent of the amount of material applied to the DEAE-cellulose column. For instance, although the amounts of albumin chromatographed varied between 84–320 mg, the total recovery was remarkably constant, *e.g.* 84.1 % ± 1.2. This is supported by the results of statistical analysis.

(b) Studies of protein mixtures

The mixtures of serum proteins investigated are tabulated in Table II where it can be seen, that with the exception of the ^{131}I labelled α_2-macroglobulin–albumin mixture, the overall protein recoveries were comparable with those shown by the individual

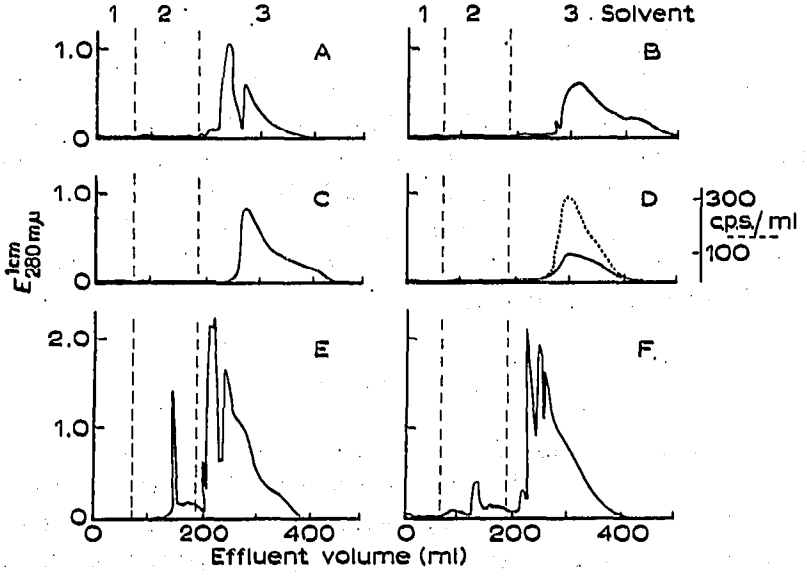

Fig. 2. The chromatographic distribution of albumin on DEAE-cellulose exchanger W. A, B and C are 82.0, 82.0 and 106.0 mg respectively of COHN F V albumin (preparation 1); D is 43.0 mg of ^{131}I labelled COHN F V (preparation 1); and E and F are 320.0 and 293.0 mg of LISTER albumin (preparation 2). For chromatographic details see text.

proteins. Moreover, the positions of elution of various components in the protein mixtures were similar to those exhibited by these proteins in isolated form. Recoveries of labelled siderophilin, and α_2-macroglobulin, which had been added to whole serum were comparable with those shown by the isolated proteins. On the other hand, the recoveries of ^{131}I labelled 7 S-γ-globulin, β-lipoprotein and albumin from whole serum were lower than the corresponding recoveries observed with isolated proteins and simple protein mixtures.

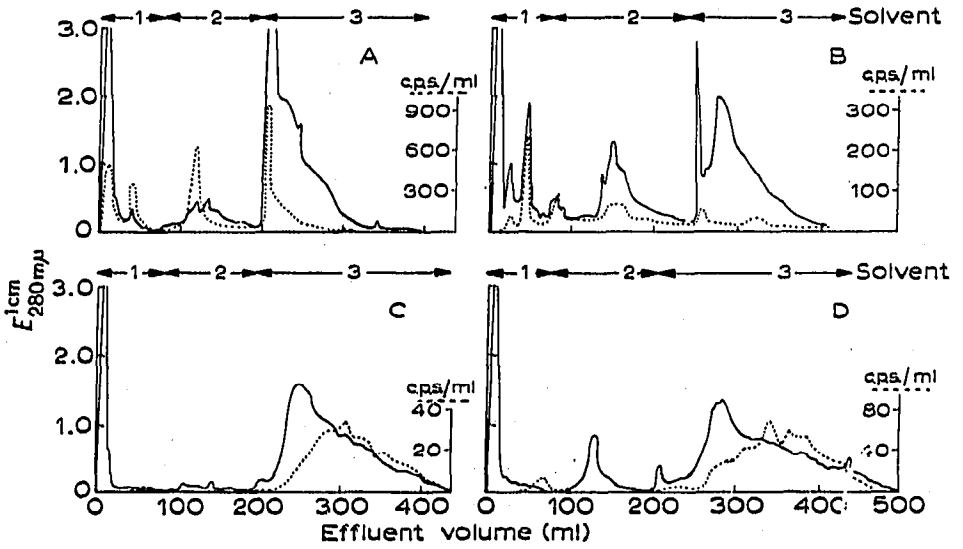

Fig. 3. The chromatography of mixtures of isotopically labelled serum proteins and whole serum on DEAE-cellulose exchanger W. The following isotopically labelled serum proteins were added to 5 ml of normal human serum: A = 6.3 mg of ^{131}I labelled 7 S-γ-globulin preparation 2; B = 141.8 mg of ^{59}Fe labelled siderophilin preparation 3; C = 12.6 mg of ^{131}I labelled α_2-macroglobulin; D = 45.7 mg of ^{131}I labelled albumin preparation 1. Distribution of radioactivity indicated thus: · · ·. For further chromatographic details see text.

In these experiments, involving the addition of labelled proteins to whole serum, the distribution of protein-bound isotope was often found to differ from that observed during the chromatography of simple mixtures containing the labelled protein. This was particularly noticeable in experiments employing ^{131}I labelled 7 S-γ-globulin (see Fig. 3), where the major portion of the labelled protein was eluted with solvents other than the 0.01 *M* phosphate buffer (pH 7.6). It should also be noted that the total recoveries of protein from isotopically labelled protein–serum mixtures were less than those obtained with serum alone.

DISCUSSION

On the whole, the chromatographic behaviours of isolated serum proteins proved similar to those exhibited during the fractionation of total serum.

As suggested from earlier studies on whole serum, the position of elution of an individual component depends largely on its charge density at the pH employed. Other factors, however, appear to influence the chromatographic separation of certain serum proteins. For example, although both α_2-macroglobulin and ceruloplasmin fall into the α_2-globulin electrophoretic class these proteins exhibit quite

distinct chromatographic properties. Similarly, there are marked differences in the chromatographic behaviour of siderophilin (a β-globulin) and 19 S-γ-globulin, in spite of their relatively small differences in electrophoretic mobility.

The results of the studies on artificial protein mixtures failed to demonstrate that protein–protein interaction was responsible for the differing behaviour of these pairs of proteins. It is more likely, however, that molecular size plays a critical role in the ion-exchange chromatography of proteins on substituted cellulose as suggested by PETERSON AND SOBER[14], resulting in the phenomenon known as "size compensation". This could explain the difference in chromatographic behaviour of 7 S-γ- and 19 S-γ-globulin. Another complication results from variations in the distribution, availability and degree of ionization of the charged groups within a protein molecule. This renders difficult the precise duplication of chromatographic separations of serum proteins. In addition, it is also possible that certain charged groups are not revealed until the protein molecule becomes unfolded on binding to the ion exchanger. Such groupings might be expected to play an active part in irreversible binding, which is assumed to be responsible for the low recoveries of proteins such as α_2-macroglobulin and β-lipoprotein.

The weak affinity of 7 S-γ-globulin for exchanger, probably due to its relatively high isoelectric point, is reflected by both its position of elution and its good recovery. Nevertheless, as already mentioned, a certain degree of trailing of the 7 S-γ-globulin was observed, successive fractions showing progressively greater electrophoretic mobility (see refs. 10 and 11). The rather abnormal distribution of ^{131}I labelled 7 S-γ-globulin (see Fig. 3A) was probably due to configurational changes produced in this molecule due to excessive iodination. Immunoelectrophoresis failed to reveal any such changes, however.

The alterations in the chromatographic behaviour of siderophilin following its isolation were puzzling. This could have been due to irreversible changes in its molecular configuration resulting from the disruption of its combination with other kinds of protein molecules. Such an irreversible change is suggested by the results of experiments involving the addition of ^{59}Fe labelled siderophilin to whole serum (see Fig. 3B), which showed that a large proportion of the added protein was still eluted prematurely. However, this effect could have been due to the use of a large excess of added siderophilin. Alternatively, structural changes not attributable directly to any protein–protein interaction effect could have played a part in the observed anomalous behaviour. The elution of the siderophilin preparations in a number of distinct peaks could have been due to the presence in the mixture of a range of siderophilin molecules saturated to varying extents with iron, or due to the existence of several distinct siderophilins[15].

The poor recoveries and "trailing" of both the β-lipoprotein and α_2-macroglobulin can be attributed to the molecular size of these proteins. Having a large partial specific volume they are susceptible to entrapment between the cellulose particles. In addition, it seems reasonable to assume that the simultaneous disruption of all the groups involved in the adsorption processes is never realised. In the case of the β-lipoprotein there is the additional complication of the high lipid content, which may render the molecule surface active, thereby causing increased affinity between exchanger and protein leading to denaturation. Moreover, this protein is rather insoluble in the aqueous eluting solvents. The elution of the α_2-macroglobulin

prior to albumin would appear to be a reversal of the size compensation phenomenon observed with 7 S-γ- and 19 S-γ-globulin. The most plausible explanation of this effect is a difference in the availability of the ionizable groups of the two proteins, for their total dicarboxylic and sialic acid contents are similar.

Because of the high dicarboxylic acid content (and hence low isoelectric point), the albumin molecule is adsorbed strongly onto DEAE-cellulose at alkaline pHs. However, this adsorption process must be readily reversible because the albumin is efficiently recovered. This could be due to the marked configurational adaptability of the albumin molecule. The appearance of albumin in a number of peaks and over a wide area of the chromatogram (see Fig. 2) has been attributed to a number of factors including the binding of small ions, especially fatty acids[16], dimer formation[17,18] facilitated by the presence of fatty acid[19,20] and to the concentration dependence of the adsorption isotherms[21]. It has also been suggested that this phenomenon might result from successive stepwise decreases in the capacity of the column under the influence of the eluting buffer[22,23]. Experiments involving the chromatography of COHN F V on DEAE-cellulose prepared from cotton linters (illustrated in Fig. 4),

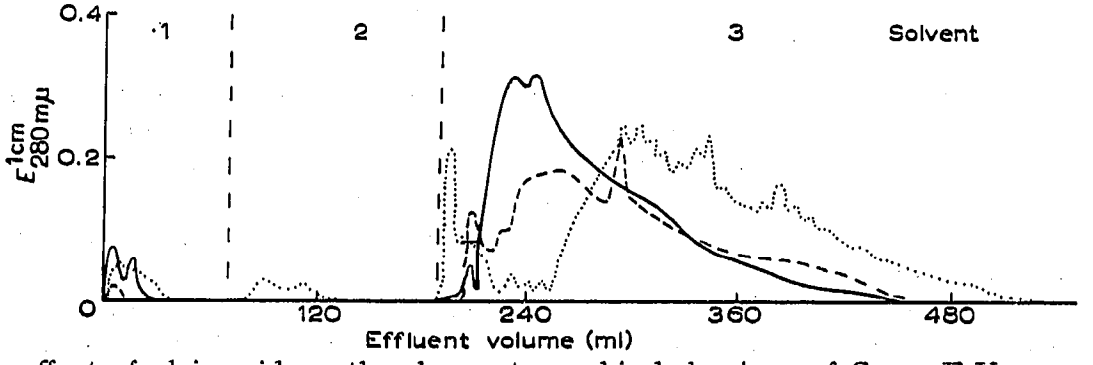

Fig. 4. The effect of oleic acid on the chromatographic behaviour of COHN F V serum albumin (preparation 1) on DEAE-cellulose (commercial exchanger 1.2 mequiv./g substitution). Chromatographic distribution represented as follows: albumin ———, ether extracted albumin – – – and oleic acid saturated albumin · · ·. For additional chromatographic details see text.

indicate that oleic acid saturated protein is more firmly bound to the column and less readily recovered than the other proteins. These results support the observations of previous workers[14,16]. Hence the binding of small, immunologically undetectable molecules, is most probably a factor of paramount importance in the observed chromatographic anomalies of human serum albumin.

As the investigation of isolated serum proteins has confirmed a wide variation in affinity for DEAE-cellulose, and has also failed to demonstrate the occurrence of any obvious protein–protein interactions, it seems probable that further studies of a similar nature could lead to the development of selective adsorption techniques for the isolation of specific serum components. Preliminary studies have demonstrated that batch chromatographic procedures, involving selective adsorption under strictly controlled conditions, could be used for such purposes.

ACKNOWLEDGEMENTS

The authors wish to thank Prof. J. R. SQUIRE for his advice and encouragement,

Dr. K. W. WALTON for supplying the COHN fractions and Mr. A. PARKER who gave valuable technical assistance. One of us (K.J.) was in receipt of a Medical Research Council Studentship for training in research methods when this work was undertaken.

SUMMARY

1. The chromatographic behaviour of several purified human serum proteins on columns of diethylaminoethyl-cellulose has been established. The group of proteins investigated included 7 S-γ-globulin, siderophilin, β-lipoprotein, α_2-macroglobulin and albumin, *i.e.* molecules with a wide range of physico-chemical properties.

2. Individual proteins were found to differ widely in their recoveries. For example, 7 S-γ-globulin and albumin were recovered in high yield in spite of their widely different affinities for DEAE-cellulose. On the other hand, a major portion of the β-lipoprotein and α_2-macroglobulin investigated (proteins of large molecular size) could not be recovered from the exchange cellulose.

3. The chromatographic characteristics of the isolated proteins closely paralleled those shown by these proteins during the fractionation of simple mixtures, though such a close relationship was not observed in whole serum. Purified siderophilin, however, proved an exception in showing a much reduced affinity for exchanger whether in isolated form or even when added to more complex mixtures.

REFERENCES

1 K. JAMES AND D. R. STANWORTH, *J. Chromatog.*, 15 (1964) 324.
2 D. R. STANWORTH, *Nature*, 188 (1960) 156.
3 E. J. COHN, L. E. STRONG, W. L. HUGHES, Jr., D. J. MULFORD, J. N. ASHWORTH, M. MELIN AND M. L. TAYLOR, *J. Am. Chem. Soc.*, 68 (1946) 459.
4 J. L. ONCLEY, M. MELIN, D. A. RICHERT, J. W. CAMERON AND P. M. GROSS, Jr., *J. Am. Chem. Soc.*, 71 (1948) 541.
5 R. A. KEKWICK AND M. E. MACKAY, *Med. Res. Council, Spec. Rept. Ser.*, No. 286 (1954).
6 A. S. MCFARLANE, *Nature*, 182 (1958) 53.
7 J. W. L. DAVIES, C. R. RICKETTS AND J. P. BULL, *Clin. Sci.*, 23 (1962) 411.
8 N. VEAL AND H. VETTER, *Radioisotope Techniques in Clinical Research and Diagnosis*, Butterworth, London, 1958, p. 255.
9 E. A. PETERSON AND H. A. SOBER, *J. Am. Chem. Soc.*, 78 (1956) 751.
10 J. L. FAHEY AND A. D. HORBETT, *J. Biol. Chem.*, 234 (1959) 2645.
11 D. R. STANWORTH, *Immunology*, 2 (1959) 384.
12 K. JAMES, *Ph. D. Thesis*, Birmingham, 1962.
13 M. D. TOMBS, K. B. COOKE, O. BURSTEN AND N. F. MACLAGAN, *Biochem. J.*, 80 (1961) 284.
14 E. A. PETERSON AND H. A. SOBER, in F. W. PUTNAM (Editor), *The Plasma Proteins*, Vol. 1, Academic Press, New York, London, 1960, p. 105.
15 E. R. GIBLETT, C. G. HICKMAN AND S. O. SMITHIES, *Nature*, 183 (1959) 1589.
16 E. A. PETERSON AND E. A. CHIAZZE, *Arch. Biochem. Biophys.*, 99 (1962) 136.
17 K. B. COOKE, M. P. TOMBS, R. D. WESTON, F. SOOTER AND N. F. MACLAGAN, *Clin. Chim. Acta*, 4 (1959) 779.
18 S. KELLER AND R. J. BLOCK, *Arch. Biochem. Biophys.*, 85 (1959) 366.
19 K. SCHMID AND A. POLIS, *J. Biol. Chem.*, 230 (1960) 853.
20 A. SAIFER, A. H. ELDER AND F. VECSLER, *J. Biol. Chem.*, 235 (1960) 1345.
21 H. A. SOBER AND E. A. PETERSON, *Federation Proc.*, 17 (1958) 1116.
22 H. G. BOMAN, *Arch. Biochem. Biophys.*, 64 (1956) 217.
23 O. LEVIN, *Arch. Biochem. Biophys.*, 78 (1958) 33.