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

I. THE EFFECT OF THE CHEMICAL AND PHYSICAL NATURE OF THE EXCHANGER

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INTRODUCTION

Although ion-exchange chromatography on substituted cellulose derivatives[1], particularly diethylaminoethyl-cellulose, has found wide application in the fractionation of serum proteins[2,3], surprisingly few detailed investigations have been undertaken to establish the factors influencing the separation of proteins accomplished by this procedure. Recently the need for such investigations have been emphasized (PORTER[4] and PETERSON AND CHIAZZE[5]).

The work to be described here was undertaken with the object of improving the effectiveness of the DEAE-cellulose chromatographic procedure as a means of fractionating serum proteins. In addition to column chromatography, a rapid batch procedure (STANWORTH[6]) has been used to study a number of the factors influencing the ion-exchange process.

Results of studies of the effect of the degree of substitution and physical form of the exchangers, and also the temperature of elution, are reported in this paper (Part 1). The standard procedure thus evolved has been employed in further studies (reported in Part 2), involving the detailed investigation of the chromatographic properties of individual purified serum proteins and artificial mixtures of these.

METHODS

Preparation of samples

Three different samples of human serum were used. Sample 1 (serum separated from a normal human donor) was used in all the column experiments with laboratory prepared exchanger.

Sample 2, used in the column chromatographic experiments on the commercial exchangers, was prepared by the defibrination of pooled acid citrate dextrose (ACD) plasma which had been stored at 4°.

Sample 3 comprised serum separated from a single donation of blood from a rheumatoid arthritic patient and was used in all the batch chromatographic experiments, providing, in addition, data on the chromatographic behaviour of rheumatoid factor.

Prior to chromatography, all the samples were dialysed against 200 volumes of the starting buffer (pH 7.6, 0.01 *M* phosphate) for 20–24 h. The samples were then centrifuged at 3,000 r.p.m. for 10 min, before application to the chromatographic columns.

Adsorbents

The DEAE-cellulose used was obtained from two sources. One sample ("W") was prepared in the laboratory from wood cellulose, according to the method of PETERSON AND SOBER[1]. This exchanger was powder-like in form and had a degree of substitution of 0.87 mequiv./g, as revealed by titration (with *N*/100 hydrochloric acid) of a suspension of the exchanger in 0.15 *M* saline. The other samples were obtained from commercial sources. These exchangers were floc-like in form, having been prepared from fibrous cotton linters. Their exchange capacities ranged between 0.05 and 1.2 mequiv./g. In this series of experiments the exchangers are referred to as C 5, C 25, C 65 and C 120, where the letter "C" indicates their cotton source and the number their degree of substitution in mequiv./g × 100.

CHROMATOGRAPHIC PROCEDURE

The exchanger "W" (2 g) was washed successively with two 50 ml volumes of 0.05 *M* NaH_2PO_4 solution and phosphate buffer (pH 7.6, 0.01 *M*) before being suspended in 30 ml of the latter buffer and poured into a column (diameter 1 cm). The column was then washed overnight with at least 100 ml of phosphate buffer (pH 7.6, 0.01 *M*), the final length of the column being about 16 cm.

Due to the floc-like nature of the "C" type exchangers, even packing of the columns by means of pouring a slurry of the exchanger into the column and applying positive pressure proved difficult. In addition, the low capacity of these exchangers necessitated the use of larger weights of material (see Discussion). The dry exchanger (3 g) was, therefore, packed a little at a time into the column (diameter 1 cm). After packing, the column was equilibrated by washing with 300–500 ml of the pH 7.6, 0.01 *M* phosphate buffer, giving a column of length between 13 and 15 cm.

The batch chromatographic experiments to be described were performed using 0.5 g quantities of exchanger "W", and 1.0 g samples of the "C" type exchangers. These samples were washed initially with 25 ml volumes of 0.05 *M* NaH_2PO_4 solution and finally equilibrated with phosphate buffer (pH 7.6, 0.01 *M*).

Throughout all the investigations, fresh exchanger was used in each experiment. On no occasion was regenerated material used. In addition all equilibrations were accomplished at room temperature, whilst chromatography was performed at 4° (except where otherwise stated).

ELUTION PROCEDURE

Column experiments

The dialyzed protein samples (volume 5 ml) were carefully applied to the top of the column by means of a Pasteur pipette and then allowed to run in under gravity. Elution was performed 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.

The flow-rates for the columns prepared from the "W" and "C" type exchangers were 3-5 ml and 60 ml per hour, respectively.

The use of an additional solvent, 0.05 *M* NaH_2PO_4 solution containing 0.25 *M* NaCl, was found to result in the elution of only a small amount of protein of very heterogeneous composition and so was not used in these column chromatographic studies. The effluent was collected in 3 ml fractions using an automatic fraction collector, incorporating a weight-balance syphon.

Examination of the effluent

The protein distribution in the effluent was determined by measuring the $E^{1\,cm}_{280\,m\mu}$ value in silica cells, in a Unicam SP 500 spectrophotometer. Effluent pH was determined by means of a micro electrode and direct reading pH meter, whilst the electrical conductivity was measured with a Mullard conductivity bridge incorporating a cathode ray (magic eye) indicator.

A standard system of pooling effluent fractions was adopted which was based upon the distribution of the main protein peaks in the chromatographic pattern of the whole serum (see Fig. 2).

Batch experiments

After equilibration, as described above for the column procedure, the exchanger ("W") was filtered through a sintered glass disc and the resultant damp exchanger was then intimately mixed with the equilibrated serum sample 3 (volume 1 ml). Following equilibration for a further 30 min, 20 ml of phosphate buffer (pH 7.6, 0.01 *M*) were added and the contents stirred to give a suspension. The sample was then allowed to stand for 30 min at 4° with frequent stirring. Exchanger and supernatant were then separated either by centrifugation, as in the case of the exchanger "W" or by filtration (see above), as in the case of the "C" type derivatives. This process was repeated with 20 ml volumes of the solvents described previously for the column procedure, but with the addition of a fourth solvent, namely 0.05 *M* NaH_2PO_4 solution containing 0.25 *M* NaCl.

In experiments investigating the effect of temperature, equilibration was performed in a thermostatically controlled centrifuge (for temperatures 0°, 10° and 22°) or in a thermostatically controlled water bath (for 37° and 50°). The equilibrated exchanger and solvent were then separated by centrifugation with minimum temperature change.

Concentration of fractions

Concentration of samples was performed at 4° by ultra-filtration through visking tubing ($^8/_{32}$ in. in diameter) using a negative pressure of 50–60 cm of Hg. After ultra-filtration adherent protein was carefully massaged from the sides of the sac and the concentrated fractions stored at —20° in polythene containers.

Analysis of fractions

Protein determinations were carried out on the concentrated fractions by the LOWRY modification[7] of the FOLIN phenol procedure, and their compositions were determined by the immunoelectrophoretic technique using a rabbit antiserum raised against human serum.

RESULTS

Column separations

Four column chromatographic fractionations of defibrinated plasma (serum sample 2) were performed on each of the "C" type exchangers. An analysis of variance indicated that there was no significant difference between the total product recovered from these exchangers at varying degrees of substitution (ranging from 0.05–1.20 mequiv./g).

For comparison, results obtained from the analysis of normal human serum (sample 1) on exchanger "W" are also included in Table I. A more direct comparison

TABLE I

THE EFFECT OF THE DEGREE OF SUBSTITUTION OF DEAE-CELLULOSE EXCHANGERS ON PROTEIN RECOVERY IN THE COLUMN CHROMATOGRAPHIC PROCEDURE

The values are given as means of four sets of results together with their standard deviations ($6\bar{x}$)

Sample	*Exchanger*	*Degree of substitution (mequiv./g)*	*Amount of protein applied (mg)*	*Protein recovery (percentage of total applied)*			
				Solvent 1	*Solvent 2*	*Solvent 3*	*Total*
				Phosphate buffer			
				pH 7.6, 0.01 M	*pH 6.3, 0.02 M*	*0.05 M NaH_2PO_4*	
Defibrinated Human A.C.D. Plasma	C5	0.05	240.6 ± 17.6	80.4 ± 6.4	3.0 ± 0.6	9.0 ± 2.1	92.8 ± 4.4
	C25	0.25	210.0 ± 15.4	56.7 ± 5.5	2.8 ± 1.1	11.1 ± 2.3	70.5 ± 6.5
	C65	0.65	265 ± 15.8	39.5 ± 4.7	9.7 ± 0.9	32.5 ± 3.8	81.6 ± 7.6
	C120	1.2	227.6 ± 6.8	13.6 ± 3.9	7.6 ± 1.5	48.6 ± 7.5	68.9 ± 2.0
Normal human serum	Laboratory prepared W	0.87	365.0	8.2 ± 2.3	7.5 ± 1.1	57.4 ± 6.8	73.0 ± 8.8

of the properties of the two types of exchangers was made, however, by employing the batch procedure (see later). In general, the total recoveries of protein from the "C" type exchanger were not significantly different from those observed with exchanger "W", although significantly greater recoveries were obtained with exchanger C5 (*i.e.* 0.05 mequiv./g).

The elution patterns varied markedly with the chemical nature of the exchanger (see Fig. 1). For instance, the amount of protein eluted with the initial solvent (phosphate buffer; pH 7.6, 0.01 *M*) was inversely related to the degree of substitution of the exchanger. Conversely, there was a direct relationship between the amount of protein recovered with the final solvent (0.05 *M* NaH_2PO_4 solution) and the degree of substitution of the exchanger.

As shown in Fig. 2 a characteristic serum elution pattern was obtained using exchanger "W", pure 7S-γ-globulin being eluted in the initial fractions. On the other hand, comparative immunoelectrophoretic analyses revealed the breakthrough of other proteins in the corresponding fractions from the "C" type exchangers. For instance, proteins such as the β- and α-globulins and the albumin, which are normally firmly bound to the exchanger at pH 7.6 (0.01 *M* phosphate), were eluted along with the 7S-γ-globulin. Consequently, the amount of protein eluted with further solvents was much reduced. Nevertheless, the protein fractions eluted with solvent 3 from these exchangers showed the heterogeneity characteristic of this part of the chromatogram (compare with Fig. 2), indicating that even ex-

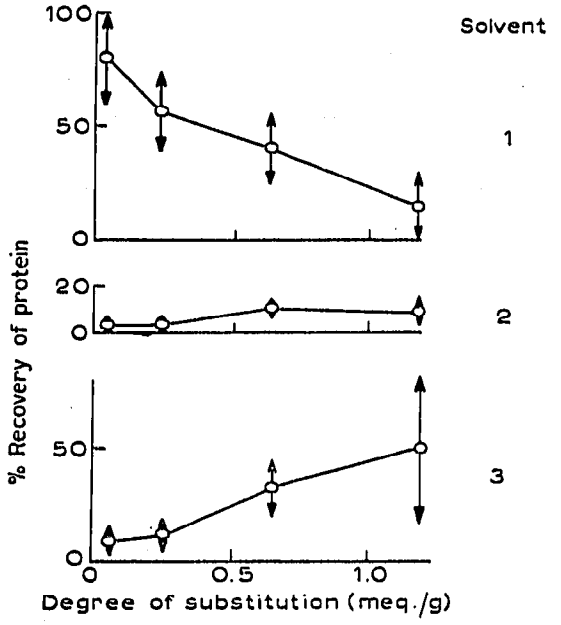


Fig. 1. The effect of the degree of substitution of "C" type exchangers on protein recoveries. The chromatographic technique employed is described in the text, whilst the amounts of protein (serum sample 2) fractionated are recorded in Table I. Each point on the graph represents the mean of four observations. The deviations illustrated by the arrows are derived by multiplying the standard deviations of the mean by the value of t at the 5% significance (0.05 probability) level for the respective number of degrees of freedom.

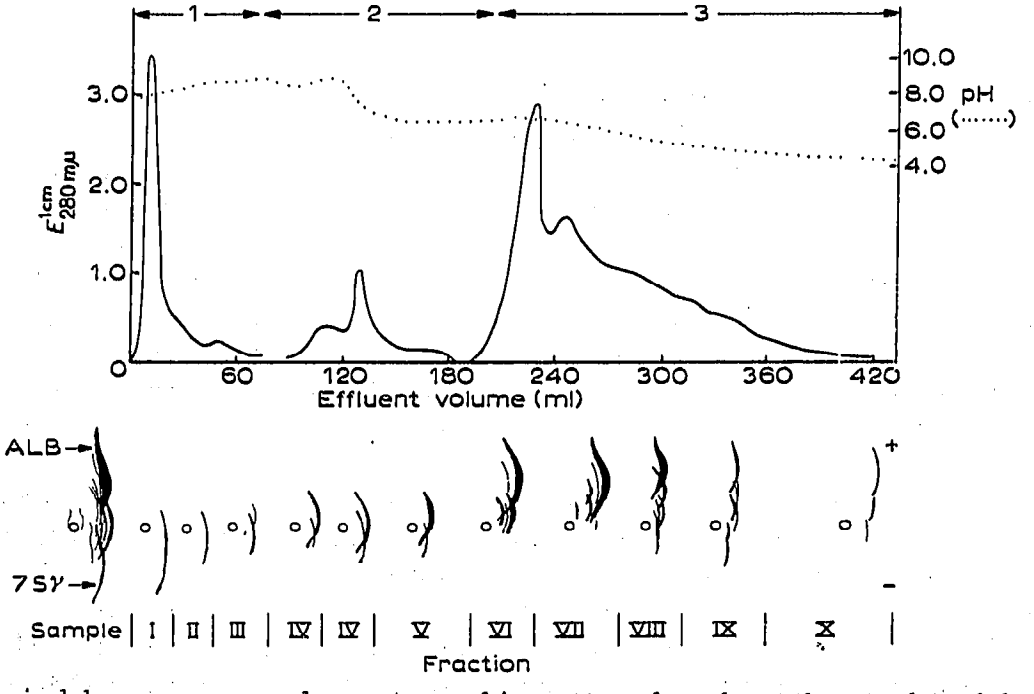


Fig. 2. A typical human serum chromatographic pattern based on the results of four different column separations of 5 ml of serum, sample 1, on type "W" exchanger. The dotted line indicates the pH of the effluent whilst the heavy line represents the protein concentration as measured by absorbancy at 280 mμ. Tracings of immunoelectrophoretic patterns obtained by testing the fractions with rabbit anti-whole human serum are also included. For chromatographic conditions see text.

TABLE II

THE EFFECT OF THE DEGREE OF SUBSTITUTION OF DEAE-CELLULOSE EXCHANGERS ON PROTEIN RECOVERY BY THE BATCH PROCEDURE

The results given are those obtained in experiments using 1 ml samples of serum 3, 1 g amounts of the "C" type exchangers and 0.5 g amounts of exchanger "W"

Exchanger	*Degree of substitution (mequiv./g)*	*Protein recovery (percentage of total applied)*				
		Solvent 1	*Solvent 2*	*Solvent 3*	*Solvent 4*	*Total*
		Phosphate buffer		*0.05 M NaH_2PO_4*	*0.05 M NaH_2PO_4 containing 0.25 M NaCl*	
		pH 7.6, 0.01 M	*pH 6.3, 0.02 M*			
C5	0.05	57.5	13.2	9.9	15.8	96.4
C25	0.25	39.3	12.9	11.9	22.5	86.6
C65	0.65	22.0	8.8	15.0	36.2	82.0
C120	1.2	28.3	6.3	13.5	46.9	95.0
W	0.87	13.0	6.0	9.3	54.5	82.8

changers of a very low degree of substitution (*i.e.* 0.05 mequiv./g) have a retentive capacity for a certain number of serum proteins.

Batch separations

In all the batch experiments performed (see Table II), there were no significant differences in the recovery of total serum protein (sample 3) from the various exchangers ("C" and "W").

These recoveries were greater than those achieved by the corresponding column procedures described above. However, the relative amounts of protein eluted with the various solvents were similar to those observed with the column technique (see Fig. 3).

As in the case of the column separations, breakthrough of proteins other than 7 S-γ-globulin occurred only in the fractions eluted from "C" type exchangers with solvent 1.

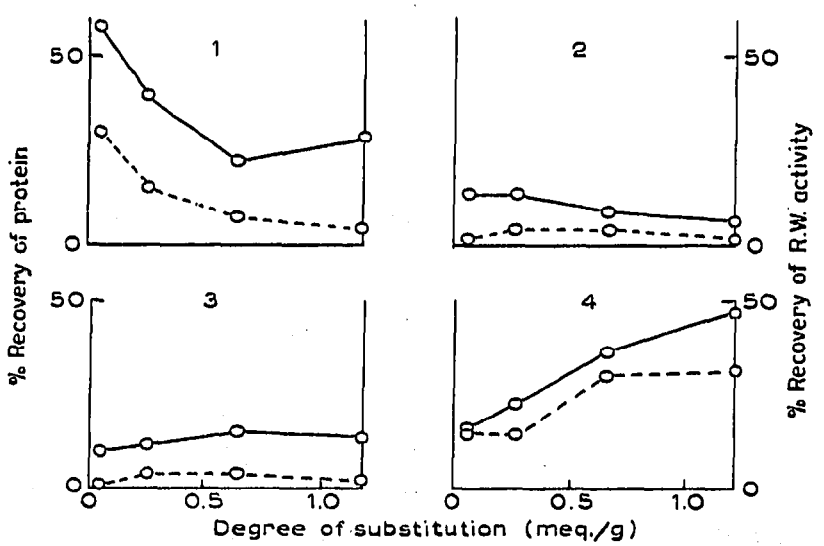


Fig. 3. Effect of the degree of substitution of "C" type exchangers on mean protein recovery (———) and recovery of ROSE-WAALER activity (– – –), during batch experiments using serum sample 3. For chromatographic conditions see text.

As the degree of substitution of the exchangers increased, the α_2-macroglobulin and albumin content in the initial effluent decreased. The major components of fractions eluted from all the different exchangers by the final solvent, however, were the α-globulins (haptoglobin and ceruloplasmin), 19S-γ-globulin and albumin, suggesting that at least these proteins were retained.

By employing rheumatoid serum (sample 3), it was also possible to study the chromatographic behaviour of a human 19S-γ-globulin, the "rheumatoid factor". This protein was detected in the effluent by means of its capacity to agglutinate sensitised sheep erythrocytes, (*i.e.* by the ROSE-WAALER technique, as modified by BALL[8]). It was hoped that the strong affinity shown by this protein for DEAE-cellulose exchangers of about 1 mequiv./g substitution, a factor which complicates its isolation on these derivatives, would be overcome by employing less highly substituted materials. In this way, it might have been possible to first selectively adsorb the rheumatoid factor and subsequently to recover it readily. Unfortunately, however, the widespread distribution of serological activity amongst the various chromatographic fractions (see Fig. 3) indicated no preferential adsorption on "C" type exchangers of relatively low degrees of substitution, thus limiting their use in the isolation of rheumatoid factor.

The effect of temperature on the chromatographic separation of human serum proteins on DEAE-cellulose was also investigated. Changes of temperature between 0–50° had a negligible effect on the specific adsorption capacity of the laboratory prepared exchangers for human serum proteins (see Fig. 4).

The amounts of protein remaining adsorbed at pH 7.6 (0.01 *M* phosphate), at

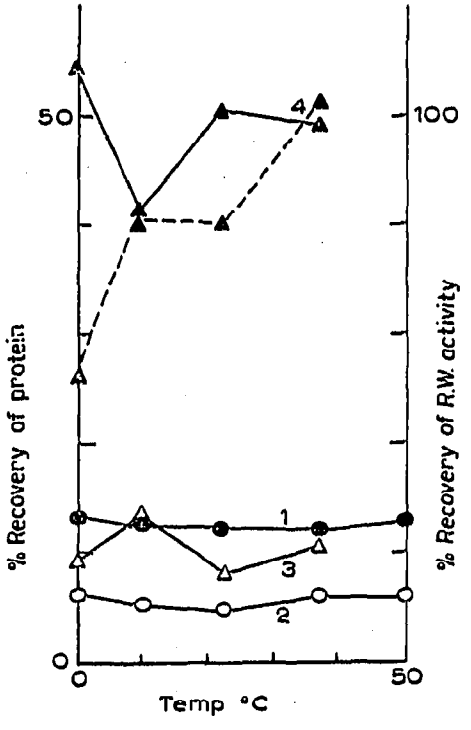


Fig. 4. Effect of temperature on the mean protein recovery (———) and recovery of ROSE-WAALER activity (– – –) during the batch chromatography of serum sample 3 on exchanger "W". Recoveries of activity with solvents 1, 2 and 3 were negligible and so have been omitted from the diagram. For chromatographic conditions see text.

the various temperatures tested, were almost identical and the effluent compositions were very similar. The quantitative and qualitative composition of the protein eluted with the other solvents were comparable, although there was some variation in the recovery of rheumatoid factor; this could, however, be partially attributed to the limitations of a doubling dilution technique of estimation of rheumatoid factor activity.

From the mean protein recoveries obtained in the above experiments, the specific adsorption capacities of the various exchangers in both the column and batch procedures have been determined and these are plotted in Fig. 5. In these experiments the "specific adsorption capacity" is defined as the total amount (mg) of serum protein adsorbed per 100 mg of exchanger, after both have been equilibrated with phosphate buffer (pH 7.6, 0.01 *M*). This permits a comparison of values obtained in these studies with the results reported by PETERSON AND SOBER[1]. As will be seen, the amount of protein adsorbed was related to the degree of substitution of the exchanger. The adsorption curves reached a plateau, above which the substitution of further reactive groupings had little effect on protein adsorption.

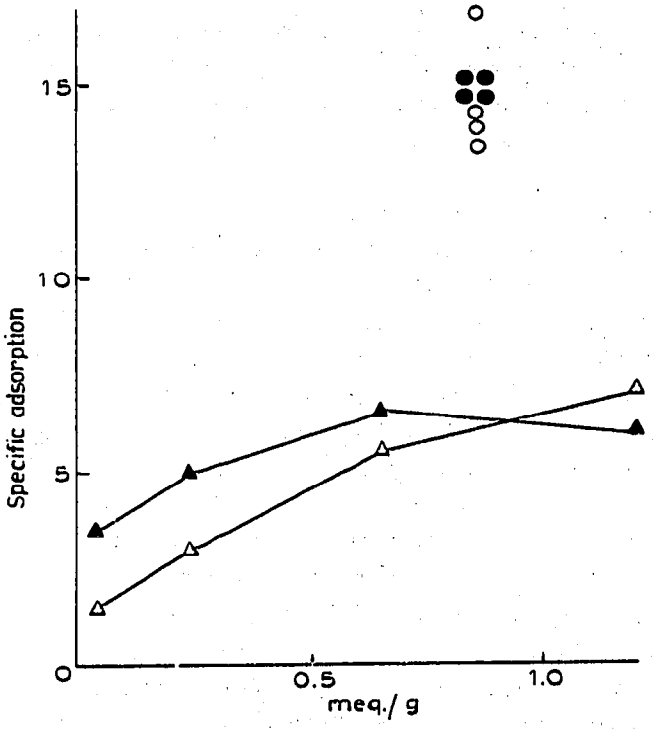


Fig. 5. A comparison of the specific adsorption capacities of "C", (–△–△–, –▲–▲–) and "W" (○, ●) type exchangers for total human serum proteins, by both column (–△–△–, ○) and batch (–▲–▲–, ●) techniques. The batch experiments on "W" type exchangers were performed between the temperatures of 0 and 50°.

DISCUSSION

Although the present study has not led to the ready development of an improved procedure for the chromatographic fractionation of human serum proteins on DEAE-cellulose, the data presented offer an indication of how various factors influence the ion-exchange process.

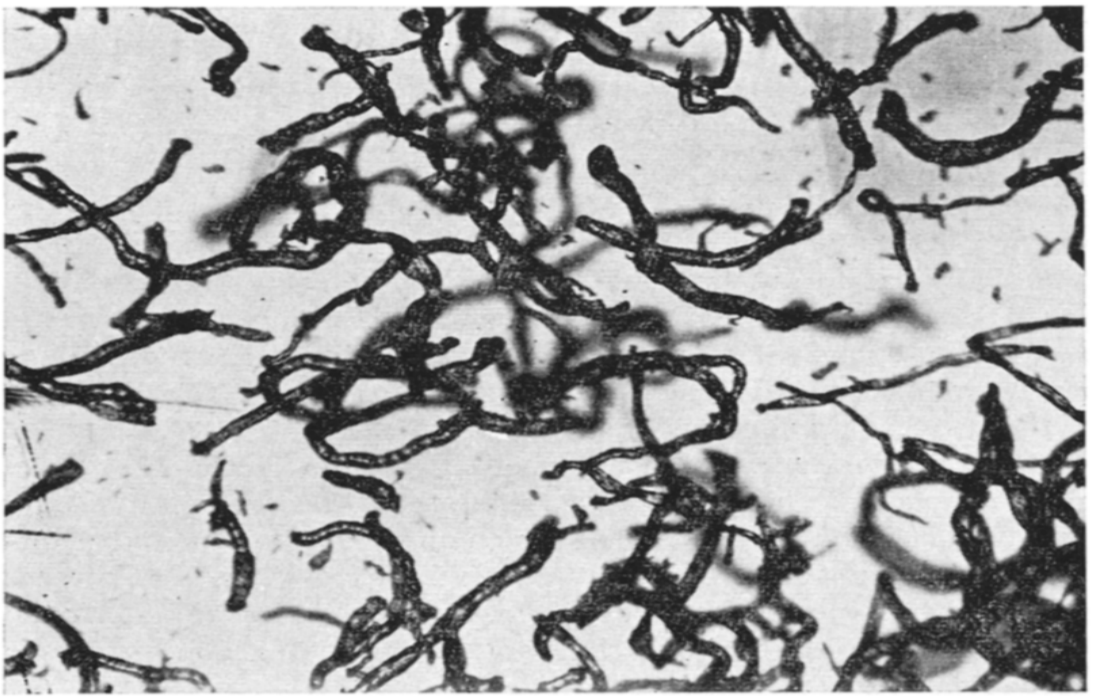
By employing exchangers of varying degrees of substitution it was hoped to

achieve the selective adsorption of certain proteins. Furthermore, by using exchangers of low degrees of substitution it should have been possible theoretically to overcome the irreversible binding which seemed to accompany the chromatography of some of the serum proteins, notably the 19 S-γ-globulins. It was found, however, that there was no selective retention of the 19 S-γ-globulin (rheumatoid factor) molecule on exchangers of low degrees of substitution (see Fig. 3). On the other hand, smaller molecules such as ceruloplasmin and haptoglobin were preferentially adsorbed under similar conditions in both the column and batch procedures. Hence it would appear that some proteins compete for the available sites on the surface of the exchanger, molecules with a relatively high affinity for the exchanger displacing those with a smaller affinity. It is possible that this property could be applied to the partial purification of specific serum proteins. For example α_2-macroglobulin can be separated from the bulk of the other serum proteins (including 19 S-γ-globulin and the other α-globulins) by the batch chromatography of serum on ion exchangers of 0.25 mequiv./g substitution or less, which had been equilibrated with phosphate buffer (pH 7.6, 0.01 *M*). Theoretically, it should then be possible to adsorb selectively the α_2-macroglobulin on an exchanger of high substitution (0.7–1.0 mequiv./g). In practice, however, it was found that recoveries by such a two-stage process were small.

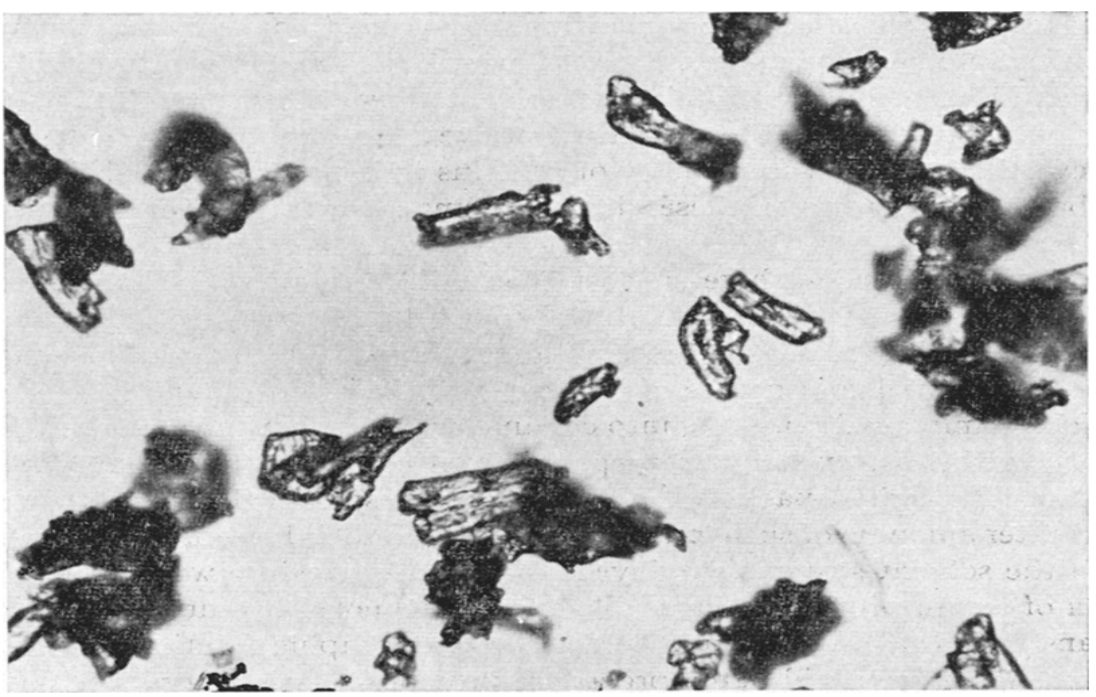
Another useful fraction rich in ceruloplasmin and other α-globulins could also be obtained by a selective adsorption procedure, *i.e.* by equilibrating a mixture of exchanger and serum with 0.05 *M* NaH_2PO_4 solution and then eluting the exchanger with a solvent of high ionic strength (such as 0.05 *M* NaH_2PO_4 solution containing 0.25 *M* NaCl). It should be mentioned, in this respect, that such selective adsorption techniques have already been applied by other investigators (*e.g.* CONNEL AND SHAW[9], STEINBUCH AND QUENTIN[10] and STEINBUCH AND LOEB[11]) to the isolation of serum α-globulins. In order to obtain further information about the relative selectivity of exchangers of differing degrees of substitution it will be necessary to study the adsorption characteristics of individual isolated serum proteins.

It is significant that the specific adsorption capacities obtained in the present series of experiments are approximately one tenth of those observed by PETERSON AND SOBER[1]. Although those investigators employed bovine serum albumin, in elutions with pH 7.0, 0.01 *M* phosphate buffers, it is difficult to explain the observed differences. Nevertheless, the two sets of results showed the same trend, the amount of protein adsorbed being proportional to the degree of substitution of the exchanger. In contrast, however, the adsorption curves shown in Fig. 5 reached a plateau above which the substitution of further reactive groupings had little effect upon protein adsorption. This is not apparent from the results of PETERSON AND SOBER, where marked increases in adsorption capacity were observed between the ranges 0.45 to 1.34 mequiv./g substitution. The higher adsorption capacity observed during the batch (as opposed to the column chromatography) procedure with "C" type exchangers of less than 0.65 mequiv./g substitution can probably be attributed to the fact that the elution with the first solvent is less efficient than in the column procedure.

As previously mentioned, some protein is adsorbed by exchangers of low substitution. For example, the exchanger C 5 adsorbs 20 % of the total amount of protein adsorbed by exchanger C 120, although its degree of substitution is only 4 % of that of the latter exchanger. This would suggest that either C 5 exhibits considerable non-ionic adsorption, or else a high proportion of the ionizing groups in the exchanger of



(a)



(b)

Fig. 6. Photomicrographs of "C" and "W" type exchangers. A. Exchanger C120, magnification × 100. B. Exchanger W, magnification × 250.

high substitution are not available for combination with the protein. It is assumed here, that the washing procedure has been efficient, and that no protein is trapped within the exchanger.

The differences in the chromatographic properties of the "W" type and "C" type exchangers (of similar substitution) can readily be explained in terms of differences in their physical form, as will be seen from the photomicrographs shown in Fig. 6. The "C" type exchangers are of a coarse physical form being prepared from fibrous cotton linters, whereas the "W" type product was crystalline-like. The effect of the physical form of DEAE-cellulose on its chromatographic behaviour has previously been discussed by PETERSON AND SOBER[1], who showed that gelatinous adsorbents (of fine physical form) possess higher capacities than non-gelatinous adsorbents. As well as affecting the capacity of the exchanger, the physical form also influences the resolution which was not so pronounced on the "C" type exchangers as in the laboratory product. A consideration of the factors which affect chromatographic phenomena on columns could explain these discrepancies (GLUECKAUF[12]). Disturbances due to non-equilibrium between protein solution and exchanger are liable to be more serious in exchangers of coarse form, for there is a greater tendency for longitudinal diffusion and channelling effects as well as less time for equilibration. All these factors tend to reduce the efficiency of the chromatographic process, although considerably higher rates of elution are obtained.

The results of the investigations of the effect of temperature are consistent with the observations of HJERTÉN[13], using calcium phosphate adsorbents. This factor was found to be of limited importance in the DEAE-cellulose chromatography of serum proteins. However, the effect of temperature may prove to be more marked in column procedures employing floc-like exchangers, where rises in temperature will increase diffusion and channelling of protein and solvent thus affecting resolution. The limitations of chromatography at temperatures above 4° are not inherent in the technique itself. Nevertheless, the susceptibility of proteins to denaturation and the risk of increased bacterial activity at these elevated temperatures necessitate fractionation at low temperatures.

The findings discussed here suggest that DEAE-cellulose exchangers prepared from cotton cellulose are inferior to those prepared from wood cellulose, at least as far as the separation of serum proteins is concerned. Ideally, the ion exchangers should be substituted to a degree of about 1 mequiv./g and should be of a physical form which permits ready packing into columns without showing a marked resistance to flow. Nevertheless, exchangers prepared from cotton cellulose have application in batch procedures where packing is not a problem, in spite of the necessity of using relatively greater amounts of such exchangers (owing to their low adsorption capacities).

With the solvent systems employed, the batch procedure was found to effect a resolution of serum proteins comparable to that achieved by the column technique. This means that when a large number of chromatographic fractionations are to be undertaken, the more rapid batch procedure provides a satisfactory alternative.

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SUMMARY

Studies have been undertaken to establish the influence of various factors on the fractionation of human serum proteins by DEAE-cellulose chromatography. The following points emerged:

1. In both column and batch procedures, the specific adsorption capacities of the various exchangers tested were related to their degree of substitution.

2. Exchangers of low degrees of substitution selectively adsorbed a number of serum proteins, especially α-globulins such as ceruloplasmin and haptoglobin, although they failed to retain preferentially rheumatoid factor.

3. The adsorption capacity of exchanger prepared from wood cellulose (of fine physical form) was twice the capacity of exchangers of similar substitution prepared from cotton cellulose.

4. Variation in the temperature of elution over a range from 0–50° had no effect on the properties of DEAE-cellulose exchanger (prepared from wood cellulose) as revealed by batch chromatographic experiments.

5. The batch technique would appear to provide a rapid alternative method to the column procedure for the chromatographic separation of serum proteins.

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