CHROM. 3898

SOLUBILITY CHROMATOGRAPHY OF SERUM PROTEINS

I. ISOLATION OF THE FIRST COMPONENT OF COMPLEMENT FROM GUINEA PIG SERUM BY SOLUBILITY CHROMATOGRAPHY AT LOW IONIC STRENGTH*

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SUMMARY

A method for chromatographic separation of serum proteins on the basis of solubility in buffers of low ionic strength has been developed. Its application to the isolation of the first component of guinea pig complement has led to a two-step process that yielded estimated recoveries of 45 % or more, with an increase in specific activity of at least 250-fold in most runs.

INTRODUCTION

Except for preliminary studies by PORATH[1] and SARGENT AND GRAHAM[2], little use appears to have been made of solubility in the chromatographic separation of proteins. With the advent of ion exchange and gel filtration chromatography, precipitation methods for protein purification have generally been neglected because of their inefficiency. The present series of reports describes several systems in which the advantages of precipitation may be combined with the efficiency of chromatography. The major advantage of precipitation methods is that they make use of conditions which are unfavorable to the action of the proteolytic enzymes present in many crude starting materials such as serum or tissue extracts; substances sensitive to the action of such enzymes may therefore be isolated with greater ease by precipitation than by other methods. Another advantage of precipitation methods over ion exchange is that problems due to the limited exchange capacity of available column packings are avoided, and thus greater quantities of material can be processed at one time.

Solubility chromatography is performed on gel columns of a sufficient degree of cross-linkage to exclude the protein to be purified. Such a gel column is equilibrated with a solution in which some or all of the proteins are insoluble. A zone of precipitated protein is formed on the column, whose packing prevents further movement of the

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precipitate. A front or gradient of solvent is then used to dissolve the proteins selectively. As each protein dissolves, it migrates into the column ahead of the solvent front by virtue of the gel filtration effect, and as a consequence precipitates again, to wait for the solvent front to redissolve it. The result is a counter-current process distributing the different proteins according to their solubilities under the conditions chosen. As there are many parameters that affect the solubility of proteins, the conditions for solubility chromatography can be varied over an extremely broad range.

In view of the above considerations, the use of a gel with a low degree of cross-linkage, *e.g.*, Sephadex G-100, as described by PORATH[1], would be expected to reduce the efficiency of separation since the method depends on the ability of the gel to exclude the protein.

The present report describes the fractionation of serum proteins by solubility chromatography at low ionic strength (μ). The next report in this series will deal with solubility chromatography at high ionic strength, in concentrated ammonium sulfate solutions.

The proteins of interest in this laboratory are the nine components of the complement (C')* system, which together constitute a small fraction of the total serum proteins. The purification of these proteins is fraught with difficulty due to the instability of some of them, and to the fact that C'1, upon activation, destroys C'4 and C'2 (ref. 3). A common first step in many purification schemes[4–7] for the C' components therefore consists in the precipitation of the euglobulin fraction at pH 5.6, $\mu = 0.02$; under these conditions, C'1 is insoluble, as are two of the other C' components, *viz.*, C'3 and C'5 (ref. 7).

The two procedures most widely used to reduce the ionic strength are dilution with distilled water and dialysis. Dilution has the disadvantage that the concentration of the soluble proteins is immediately decreased to a considerable extent, which adds to the difficulty of subsequent purification efforts. The disadvantage of dialysis is that it requires time, during which the precipitate forms slowly and remains in contact with the proteins in the supernatant fluid. The variable yields of C'2 obtained by the method of BORSOS *et al.*[4] can probably be ascribed to inactivation at this point. By solubility chromatography, both of these difficulties can be overcome: On the one hand, dilution of the supernatant fluid is minimized; on the other, the precipitate is separated from the soluble proteins as it forms. FJELLSTRØM[8] has used this method to prepare serum reagents lacking C'1 or C'2 (R1 or R2, respectively[9]), but has apparently not used it to purify C'1.

This report describes the separation of those proteins that are soluble at pH 5.6, $\mu = 0.02$, from those that are insoluble under these conditions on a column of polyacrylamide (Bio-Gel P-10, Bio-Rad Labs., Richmond, Calif.); this gel was chosen to avoid the possibility of reaction of C' components with natural antibodies to dextran

* The components of C' are designated by numbers and are defined in terms of chemical properties and sequence of action on sheep erythrocytes (E) that have previously been treated with antibody (A), forming EA. Thus, C'*j* will denote the j^{th} component of C' according to the definition adopted by the 3rd Complement Workshop[24]. C'*j*a denotes the activated form of C'*j*, which is produced during its reaction with EA, and in some cases, by other manipulations. Intermediates in the sequence by which the C' components act on EA to produce lysis are named EAC'*j*,*i*,*k*,...; the numbers in this expression designate which C' components have reacted with the EA, and imply that C' components that are not listed must be supplied in order to lyse the cells.

which might bind to Sephadex. The average recovery of C'2 in the soluble fraction is 90%. The precipitated C'1 is eluted with a gradient of rising NaCl concentration at pH 5.6; it is recovered in high yield, purified 40-fold. The observation of NELSON *et al.*[7] that C'1 also precipitates at low ionic strength at pH 7.5 is utilized to purify C'1 further from this fraction by solubility chromatography near this pH. These efforts have yielded the surprising result that C'1 exists in two active forms of different solubilities at pH 7.7 (ref. 10).

MATERIALS AND METHODS

Apparatus

The chromatograms shown were obtained by recording the pH, optical densities, and electrical resistance of column effluents automatically. To this end, the effluent was passed successively through a flow-through conductivity cell connected to a recording conductivity bridge (Conductolyser, LKB Instruments), through a micro flow-through cuvette (Pyrocell Mfg. Co., Westwood, N.J.) in a spectrophotometer programmed to record optical densities at three selected wavelengths (Beckman Model DB-G with Programmer), and through a flow-through pH electrode system (Beckman No. 46850) connected to a pH meter (Beckman Expandomatic). Before entering the pH electrode assembly the liquid stream was interrupted by a simple device, shown in Fig. 1, to prevent grounding the glass electrode voltage to the conductivity cell. In spite of this precaution, the pH trace showed regular low-frequency oscillations with an amplitude of about 0.1 pH, which were averaged in the figures shown below. The conductivity cell was maintained at 0.7° in a thermoelectrically cooled bath. The output of the spectrophotometer, which is linear in percent transmission, was applied to a logarithmic amplifier to provide a signal linear in optical density (O.D.). The output signals from all these devices were adapted for input to a six-channel, 10 mV potentiometric recorder (Bristol Multiple-Point Dynamaster),

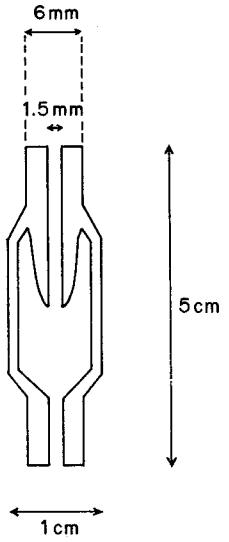

Fig. 1. Design of a stream interruptor for on-stream pH measurement.

equipped with an event marker for the fraction collector. Solutions were pumped through the columns with a peristaltic pump (LKB Instruments) to provide reasonably constant flow rates. All operations were performed in a cold room at 2–4°.

Assay procedures

The isotonic NaCl–Veronal buffer and the hemolytic intermediate EAC'1a,4 were prepared, and C'2 assays were performed, as described by KABAT AND MAYER[11]; the latter procedure was modified as described by HOFFMANN *et al.*[12]. The EAC'1a,4 preparations used in this work had t_{max} values[11] of 10–15 min. The results of C'2 assays were evaluated in accordance with the dose response relation described by HOFFMANN AND MEIER[13].

The intermediate EAC'4 was prepared by a modification[14] of the method of BECKER[15]. EAC'1a,4 were suspended in buffer containing 10 m*M* EDTA to a concentration of *ca.* 10^9 cells/ml and incubated 30 min at 37°. The suspension was centrifuged for 10 min at 1290 × *g* (at r_{max}) and the supernatant fluid was removed by suction. The cells were resuspended as before in buffer containing 10 m*M* EDTA and the incubation, centrifugation, and removal of the supernatant fluid were repeated. The cells were then washed, successively, once in buffer containing 10 m*M* EDTA and twice in buffer containing 0.15 m*M* $CaCl_2$ and 1 m*M* $MgCl_2$. Each time the cells were centrifuged, the supernatant fluid was removed, and the cells were resuspended to a concentration of *ca.* 10^9 cells/ml. They were finally suspended in buffer containing Ca^{2+} and Mg^{2+} as above, and stored at 0°.

C'1 assays were performed as described by HOFFMANN *et al.*[12]. The diluted C'1 samples were mixed with the EAC'4 immediately after each dilution was prepared. For assays of C'1 in whole serum, EAC'4 was incubated with the diluted serum sample for 3 h at 37.0° to permit complete activation of the C'1 (ref. 16). The C'2 preparations used in these assays were made by a new procedure to be described in another report; the same results are obtained with the new preparations as were obtained with C'2 lots made according to BORSOS *et al.*[4].

Electrophoretic procedures

Disc electrophoresis was performed in running gels of 7.5 % polyacrylamide at pH 9.5 (ref. 17), with a current of 5 mA per tube in a commercial apparatus (Shandon Scientific Co., Sewicky, Pa.). Since difficulty was encountered in getting the sample gels containing protein to polymerize, the samples (50 μl) were applied in solution containing *ca.* 50 % sucrose, dry polyacrylamide beads (Bio-Gel P-10, 200–400 mesh) being added to each tube to prevent convection[18]. Electrophoresis was continued until the Bromophenol Blue marker dye had just migrated to the end of the column. Stained disc electrophoresis patterns were scanned on a Gilford Model 240 spectrophotometer at 550 nm.

Immunoelectrophoresis was performed in 0.75 % agarose gel in barbital buffer, pH 8.6, $\mu = 0.05$, for 90 min at 5 mA per slide in an apparatus based on SCHEIDEGGER's procedure[19] (Agafor, National Instrument Labs., Rockville, Md.). The patterns were developed with a commercial antiserum to whole guinea pig serum (Immunology, Inc., Glen Ellyn, Ill.). Each sample analyzed by either electrophoretic procedure was adjusted to an O.D. of 3.0 (1 cm light path) at 278 nm. A constant current power supply was used for all electrophoretic procedures (Buchler No. 3-1014).

Chromatography

Column 1. A borosilicate glass column (7.7 cm I.D. × 90 cm) with removable end pieces, fitted with an upward flow adaptor (Glass Engineering Co., Houston, Tex.) was coated with dimethyldichlorosilane and charged with 300 g of polyacrylamide gel, Bio-Gel P-10, 50–150 mesh (both from Bio-Rad Labs., Richmond, Calif.), from which the fine particles had been removed by repeated decantation. The bed so obtained was 46 cm high during operation of the column. Between successive runs, the bed was stirred up completely in an excess of 0.30 *M* NaCl containing 0.01 *M* ethylenediamine tetraacetate (EDTA), pH 8.5; part of the gel was removed from the column temporarily in order to achieve complete resuspension. The gel was allowed to settle and the cloudy supernatant fluid, containing presumably undissolved protein from the previous run, was siphoned off. The column was then equilibrated by upward flow with sodium acetate-acetic acid buffer, pH 5.6, $\mu = 0.020$, at *ca.* 200 ml/h.

Fresh frozen guinea pig serum was either purchased commercially (Immunology, Inc., Glen Ellyn, Ill.) or obtained through the generosity of Dr. KENNETH AMIRAIAN, Division of Laboratories, New York State Department of Health, Albany, N.Y. The commercial serum was separated from blood obtained by cardiac puncture and contained higher concentrations of protein (O.D. at 278 nm) and hemoglobin than the serum from New York, which was separated from blood obtained by exsanguination of guinea pigs under CO_2 anesthesia[20]. This difference affects the chromatographic results as will be discussed in detail below.

Approximately 200 ml of fresh guinea pig serum, which had been stored at −65°, were adjusted to pH 5.5 with 0.15 *M* acetic acid in an ice bath. In this operation, the pH tends to drift back to more alkaline values; adjustment was continued until this drift was less than 0.1 pH in 1 min. This is of cardinal importance to the success of this chromatographic procedure, for too high a pH leads to premature elution of part of the C'1.

The adjusted serum was pumped onto the column at a rate that just prevents a build-up of serum above the top of the bed, followed by a gradient generated by a 9-chamber variable gradient former (Buchler "Varigrad"); the solutions placed in each of the chambers are shown in Table I. The gradient was followed by a 0.30 *M* NaCl solution containing 10 m*M* EDTA, pH 8.5; this step is designed to remove protein that is not soluble anywhere in the gradient, and to destroy traces of C'1 that might be left on the column. A flow rate of 300 ml/h was achieved. Twenty-milliliter fractions were collected by volume (Gilson Medical Electronics fraction collector

TABLE I

GRADIENT PROGRAM FOR COLUMN 1

Each chamber contained 140 ml of the indicated solution, adjusted to pH 5.6 with acetic acid before being made up to volume.

Component	*Concentration in chamber*								
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
Na^+-acetate, *M*	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.020
NaCl, *M*	0.000	0.018	0.035	0.050	0.180	0.140	0.010	0.400	0.300
$CaCl_2$, m*M*	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50

Model VL); all tubes beyond the expected position of the excluded (soluble) protein peak contained 1 ml of 1 *M* Tris–HCl buffer*, pH 7.5. In this manner, both the pH and ionic strength of each fraction were raised immediately upon collection to keep the proteins in solution. Electrical resistance was recorded across a flow cell with cell constant 0.704 cm^{-1} (value supplied by manufacturer), and conductivities were computed from this figure and the recorded resistance values. A flow cell with a 5 mm light path was used to record the optical densities.

The fractions containing C'1 were combined and sufficient 3.00 *M* NaCl was added to the pool to increase the NaCl concentration by 0.15 *M*. This was done to compensate for the loss of salts which occurs during subsequent ultrafiltration at high pressure (95 lb./sq. in. of N_2) through a "Diaflo" UM-1 membrane of 3 in. diameter (Amicon Co., Lexington, Mass.); this loss of salts is probably an osmotic pressure effect. Concentration of the 550-700 ml of pooled material to 20-30 ml required 16–23 h; this operation was carried on in the cold room. Recently a PM-30 membrane was substituted for the UM-1 membrane; this reduced the time required for concentration to 10–15 h. An unidentified substance absorbing ultraviolet light was regularly found in the effluents from UM-1 membranes; it had an absorption maximum at 288 nm and a minimum at 260 nm, with $A_{288}/A_{260} = 1.9$. Tests for C'1 activity in the effluents were negative.

Column 2. A borosilicate glass tube of 5.0 cm I.D. with a removable Teflon end piece (Glass Engineering Co., Houston, Tex.) was coated with dimethyldichlorosilane. One hundred grams of polyacrylamide gel (Bio-Gel P-10, 200–400 mesh) were allowed to swell in distilled water. Approximately 25 % of the gel was removed as fine particles in the course of numerous cycles of resuspension, settling, and removal of the supernatant fluid by suction. The remaining gel was packed into the column to produce a bed height of 29 cm under operating conditions. The column was equilibrated with a solution containing 10 m*M* Tris–HCl, pH 7.0 (measured at room temperature) and 0.5 m*M* $CaCl_2$. This produced a pH of 7.7 at 2°, presumably because of a shift in the pK of the buffer.

A linear gradient was formed by means of two identical cylindrical vessels in hydrostatic equilibrium; the mixing chamber initially contained 80 ml of the Tris–HCl–$CaCl_2$ solution with which the column had been equilibrated, while the reservoir contained 80 ml of a solution of 10 m*M* Tris–HCl, pH 7.0 (measured at room temperature), 0.5 m*M* $CaCl_2$, and 0.15 *M* NaCl. This gradient was pumped into the column immediately *before* the concentrated C'1 pool from Column 1. This, in turn, was followed by the starting Tris–HCl–$CaCl_2$ buffer. Flow rates between 20 and 37 ml/h were obtained in the various runs on this column for which results are presented in this report. Fractions of 5 ml were collected by volume into 0.25 ml of 1.00 *M* NaCl (Beckman/Spinco fraction collector). Recording conditions were the same as for Column 1, except that a cuvette with a 10 mm light path was used to record the O.D.

RESULTS

Solubility chromatography at pH 5.6

Fig. 2 shows a chromatogram obtained with Column 1. Optical densities were recorded at 278, 360, and 412 nm. The first peak, representing proteins soluble at

* Tris = Tris-(hydroxymethyl)-aminomethane.

pH 5.6, $\mu = 0.02$, contains heme proteins whose O.D. at 412 nm is greater than that at 360 nm. The effluent emerging later in the run is turbid; this is reflected in an O.D. at 360 nm which is greater than that at 412 nm. All fractions in the early protein peak with an O.D. at 278 nm greater than 2.0 were pooled on the basis of the recorded O.D.; they represent *ca.* 70 % of the applied protein. This pooled material frequently becomes slightly turbid at 0°; it is therefore centrifuged 1 h at 10,000 × *g* at 0°. It is then processed for C'2 production as will be described in the next report in this series.

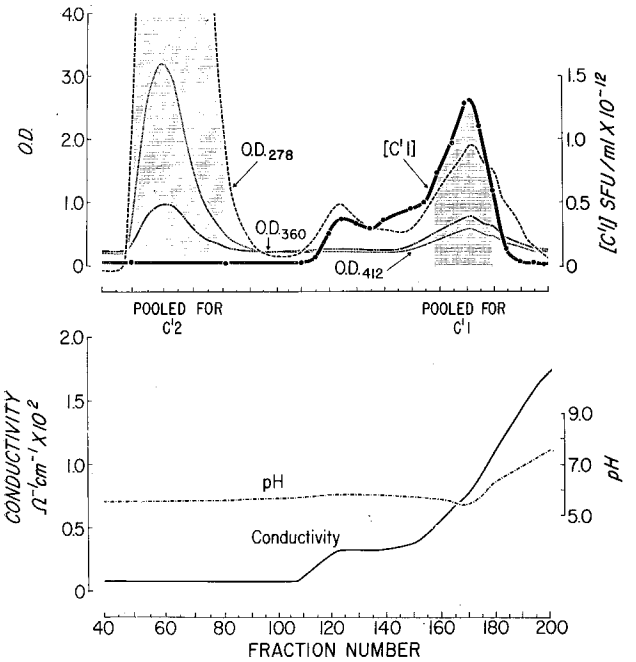

Fig. 2. Solubility chromatogram of 200 ml of whole serum at pH 5.6 and low ionic strength. Fractions: 20 ml. O.D. values are corrected to a 1 cm light path.

Some protein, containing a small amount of the C'1, is eluted in the fractions (No. 110 to 135) which contain the salts present in the applied serum. The size of the column in relation to the volume of serum applied is purposely chosen to be considerably larger than would be needed for a simple buffer change in order to allow these salts to be diluted by diffusion. If the pH adjustment of the serum before application to the column is not performed properly as described above, the pH of these fractions rises above 6.0 and considerable C'1 is eluted in this peak. Most of the C'1 emerges later in the gradient.

The chromatogram shown in Fig. 2 is representative of those obtained with commercial guinea pig serum. Chromatograms for serum from the New York State Department of Health are different in two respects: (i) The single, skewed peak of material absorbing at 412 nm with a maximum at fraction 60 in Fig. 2 is replaced by two well-defined peaks with O.D. maxima below 1.0, the first of which occurs at fraction 60, the second at fraction 80. The second peak is apparently only partially soluble at pH 5.6, $\mu = 0.020$, and thus emerges later than the soluble proteins. It

differs further from the first peak in its absorption spectrum, having a much higher ratio of $O.D._{412}/O.D._{360}$. Additional chromatographic work, incidental to studies to be reported elsewhere, has shown that these two substances also differ in solubility in concentrated ammonium sulfate solutions and in molecular size. (ii) The $O.D._{278}$ of material emerging in the gradient continues to rise past the maximum shown in Fig. 2 at fraction 170 and reaches a peak of 2.6 at fraction 185, after the C'1 is eluted. Since in later runs, fractions for the C'1 pool were selected on the basis of the O.D. pattern rather than after assay, this fact led to an error in pooling fractions from Run XXXV, the first made on serum from the New York State Department of Health; this error lowered both the apparent yield and recovery values (*cf.* Table II).

The extent of purification was estimated on the basis of absorbance, A, at 278 nm measured in a Zeiss PMQ-II spectrophotometer. Since part of the O.D. of a turbid sample at this wavelength (λ) is due to light scattering, use was made of the fact that in spectral regions outside absorption bands, all of the O.D. is due to light scattering and is proportional to λ^{-4}. Spectra in the visible range to 600 nm were therefore taken of all turbid samples; the O.D. values were plotted on log-log paper against λ and the linear portion of each such graph was extrapolated to 278 nm to estimate the light scattering contribution to the O.D. at that wavelength; usually, this represented about 60 % of the O.D. for the pooled C'1 from Column 1. The remainder of the O.D. after subtraction of the light scattering contribution was taken as A_{278}.

The results of 11 successive runs are shown in Table II. The soluble fraction shows generally high recoveries of C'2; the only notable exception was Run XXXIII, in which attempt was made to remove turbid material from the serum after adjustment to pH 5.5 by high-speed centrifugation; this led to both mechanical loss and inactivation, and the practice was abandoned. No explanation is available for the low recovery in Run XXXVII. The amount of C'1 remaining in this fraction is usually

TABLE II

RESULTS OF SOLUBILITY CHROMATOGRAPHY AT pH 5.6

Run No.	*Soluble fraction*			*C'1 pool*	
	C'2		*C'1*		
	% recovery	*Purification*	*% remaining*	*% recovery*[a]	*Purification*[a]
XXVIII	90	1.3×	0.8	460	154×
XXIX	99	1.8×	<0.05	360	105×
XXX	97	1.4×	0.042	124	47×
XXXI	103	1.3×	0.33	104	41×
XXXII	92[b]	1.2×	0.04	139	41×
XXXIII	82[b]	1.1×	0.002	82	35×
XXXIV	90	1.2×	<0.02	107	53×
XXXV	93	1.2×	<0.02	44[c]	19×[c]
XXXVI	88	1.2×	0.05	95	43×
XXXVII	77	1.1×	<0.004	263	104×
XXXVIII	93	1.3×	<0.02	238	86×

[a] Assays of C'1 pool and starting material not strictly comparable; *cf.* text.

[b] Corrected for mechanical losses of 18 % in XXXII and 45 % in XXXIII, due to fraction collector failures.

[c] Mechanical loss; see text.

well below 1 % of input; in a number of runs, it was undectable. The results on apparent recovery and purification of C'1 in the C'1 pool vary considerably. This variation can probably be ascribed to uncertainties in the C'1 assay for whole serum, which derive from the facts that an inhibitor of C'1 is present in whole serum, and that the dose response of C'1 in serum is non-linear[21], in contrast to that of isolated C'1. All that can be said is that the yields are substantial; if the relative activities of whole serum and pooled C'1 are adjusted to give a yield of 100%, the extent of purification averages 40-fold, with individual values between 29- and 50-fold.

The ultrafiltration step yields consistently high recoveries, often exceeding 100 % of the activity contained in the pooled C'1. This is probably due to the reversible, concentration-dependent dissociation of C'1 into inactive subunits described by COLTEN *et al.*[22]. If the pooled C'1 is in a partially dissociated state, the 20-fold increase in concentration during ultrafiltration should reverse that dissociation.

The total time required for a normal run on this column is 11 h; elution of the soluble fraction is complete in 4½ h, that of C'1 in 10 h.

A typical chromatogram obtained with Column 2 is shown in Fig. 3. Most of the protein emerges with the void volume of the column. This fraction always contains some C'1 activity, which has been labeled Type A, and represents a form of C'1 which is soluble at $\mu = 0.0105$, pH 7.7. If the C'1 from Column 1 is concentrated and applied to Column 2 without delay, most of the C'1 emerges in the gradient; this C'1 fraction has been designated as Type B. The conductivity corresponding to the maximum in the C'1 activity curve shows some variation from run to run; this may be due to variations in volume and salt concentration in the applied sample. In most runs, the

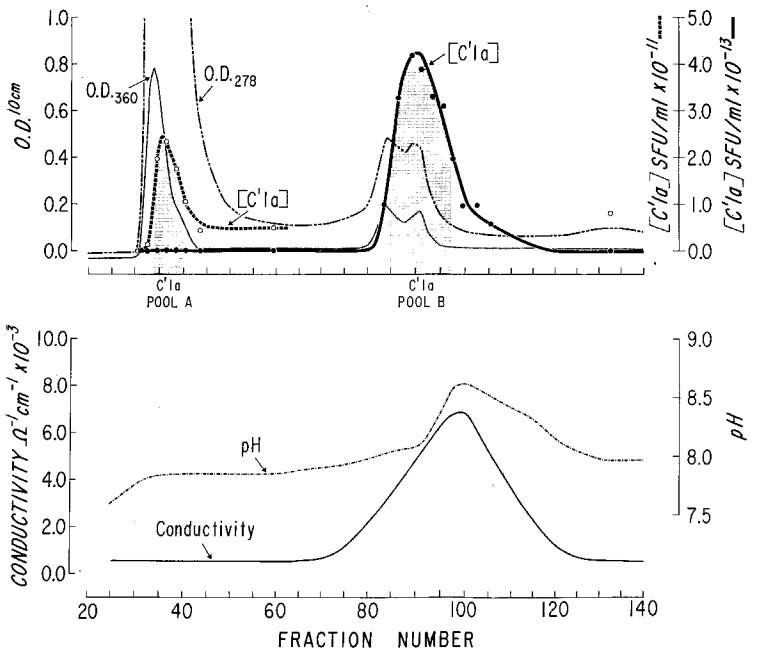

Fig. 3. Solubility chromatogram of pooled, concentrated C'1 from Column 1 (Fig. 2) at pH 7.7 and low ionic strength. Fractions: 5 ml.

conductivity at that point in the leading edge of the C'1 zone where the C'1 concentration attains half its maximal value has been fairly reproducible; it is equivalent to an NaCl concentration of 0.076 ± 0.006 *M*. The 60 ml of effluent following this point in the conductivity curve contain the bulk of the C'1. Occasionally, the C'1 emerges earlier in the gradient; no explanation is available for this fact.

The chromatographic behavior of the two types of C'1 is reproducible when each is chromatographed separately under the same conditions[10]. The present findings differ from those described in the earlier report[10] in that at that time, most of the C'1 recovered was Type A; this is due to instability of Type B C'1 in the concentrated preparation obtained from Column 1; thus, if application to Column 2 is delayed, loss of Type B occurs. This loss is not accompanied by a corresponding increase in Type A[21].

A preliminary report on the different biological properties of the two types of C'1 has appeared[23]. Type B has all the properties usually ascribed to C'1a; Type A lacks some of these properties and closely resembles the subunit, C'1s, of human C'1 as described by NAGAKI AND STROUD[24]. No Type A C'1 is recovered when whole guinea pig serum is subjected to solubility chromatography at pH 7.7; what causes its formation, presumably during operation of Column 1, is not known. Partially purified guinea pig C'1, obtained by other methods[6], can also be separated into Types A and B by the procedure described here[25]. A detailed report on the biological and physical properties of the two types of C'1 is in preparation.

Experience with eight successive runs on Column 2 is summarized in Table III. The recoveries of Type A are quite variable and tend to be lower for Runs XXXV and up, *i.e.*, for those performed with serum obtained from the New York State Department of Health. Purification of Type A is less than 1-fold because most of the protein contained in the concentrated C'1 from Column 1 emerges with this fraction, which contains only a small portion of the applied C'1. For most runs, the recovery of Type B ranges between 40 and 60 % of input, at 6- to 10-fold purification. No explanation is available for the anomalously high recovery in Run XXXII, nor for the low recovery in Run XXXV. The latter has been confirmed by repeated assay; the only unusual feature in this run was that C'1 application to the column began after 84 % of the gradient had entered the column, instead of the usual 93 %. The 101 % recovery in Run XXXVI may reflect variation in assay results between lots of EAC'4 of different ages. The normal running time for this column is 24–36 h.

TABLE III

RESULTS OF SOLUBILITY CHROMATOGRAPHY AT pH 7.7

Run No.	*Peak A*		*Peak B*	
	% *Recovery*[a]	*Purification*[a]	% *Recovery*[a]	*Purification*[a]
XXXI	5.8	<1×	43	2.4×
XXXII	13	<1×	309	6.8×
XXXIII	2.4	<1×	57	8.0×
XXXIV	3.0	<1×	45	9.8×
XXXV	0.16	<1×	9.6	6.0×
XXXVI	0.46	<1×	101	9.2×
XXXVII	0.51	<1×	82	13.0×
XXXVIII	0.17	<1×	41	5.2×

[a] Recoveries and purification both relative to material applied to the column.

Results for the combined operation of the two chromatographic procedures are shown in Table IV as recoveries and purification ratios with respect to whole serum. These figures are subject to the difficulties arising from assays on whole serum, discussed in connection with the data in Table II. Subject to qualifications arising from this source, it would appear that the procedures described will yield at least 30 to 50 % recovery of Type B C'1 at 250- to 300-fold purification.

TABLE IV

OVER-ALL RESULTS FOR BOTH STEPS: TYPE B C'1

Run No.	% *Recovery*	*Purification*
XXXI	45	98×
XXXII	430	280×
XXXIII	48	280×
XXXIV	36	250×
XXXV	6.7[a]	170×
XXXVI	174	710×
XXXVII	203	1350×
XXXVIII	77	690×

[a] In part reflects mechanical loss; see text.

The results of electrophoretic analyses of the various fractions obtained are shown in Figs. 4 and 5, the former representing tracings of scans of disc electrophoretic patterns, the latter reproducing the stained immunoelectrophoresis slides. They show that qualitative changes in composition have occurred as a result of these chromatographic procedures; this is consistent with the changes in specific activity of C'1 shown in the tables.

Comparison of the disc electrophoresis patterns for whole serum (A) and the fraction soluble at pH 5.6, $\mu = 0.02$ (B) shows two major differences: The band just after albumin, and the sharp band which barely enters the separating gel, which are seen in the pattern for whole serum, are absent in the soluble fraction. Analyses at lower protein concentration show that a small amount of the faster protein does, in fact, remain in the soluble fraction, but is obscured here because of excessive spreading of the albumin band. These two bands both appear at a considerable increase in relative concentration in the pooled C'1 from Column 1 (pattern C); however, the faster of these two bands did not penetrate the separating gel as deeply as did its apparent counterpart in pattern A, and this identification must remain tentative. The pattern for C'1 Pool A from Column 2 (D) does not differ appreciably from that of the parent fraction (C), except that the faster of the two main peaks in pattern D moved even more slowly than its counterpart in pattern C. All of the samples were subjected to electrophoresis simultaneously, and the observed mobility differences are greater than those found in replicate patterns for whole serum. The pattern for C'1 Pool B from Column 2 (E) shows one major band of very low mobility, consistent with the high molecular weight of C'1 (ref. 26) ,which would prevent this molecule from penetrating very far in the 7.5 % polyacrylamide gel.

The immunoelectrophoretic patterns generally confirm the observations made by disc electrophoresis. Patterns A and B, for whole serum and the fraction soluble

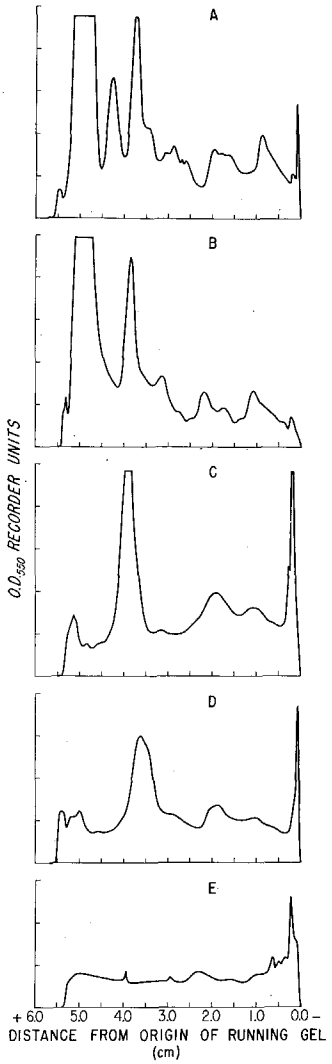

Fig. 4. Disc electrophoresis patterns of whole serum and fractions obtained by solubility chromatography. Scan A: whole serum; scan B: soluble fraction from Column 1; scan C: pooled C'1 from Column 1; scan D: C'1 Type A from Column 2; scan E: C'1 Type B from Column 2, examined at one-half the protein concentration of the other samples.

at pH 5.6, $\mu = 0.02$, respectively, are very similar, as are patterns C and D, for pooled C'1 from Column 1 and C'1 Pool A from Column 2, respectively. A notable exception is the prominent band with a mobility slightly less than that of albumin, which is seen in the disc electrophoretic patterns for whole serum, pooled C'1 from Column 1, and C'1 Pool A from Column 2; no arc corresponding to this protein is found in the immunoelectrophoresis patterns. This may be due to the absence of an antibody to this constituent in the antiserum used. The patterns for pooled C'1 from Column 1 (C) and for C'1 Pool A from Column 2 (D) bear little resemblance to the pattern for whole

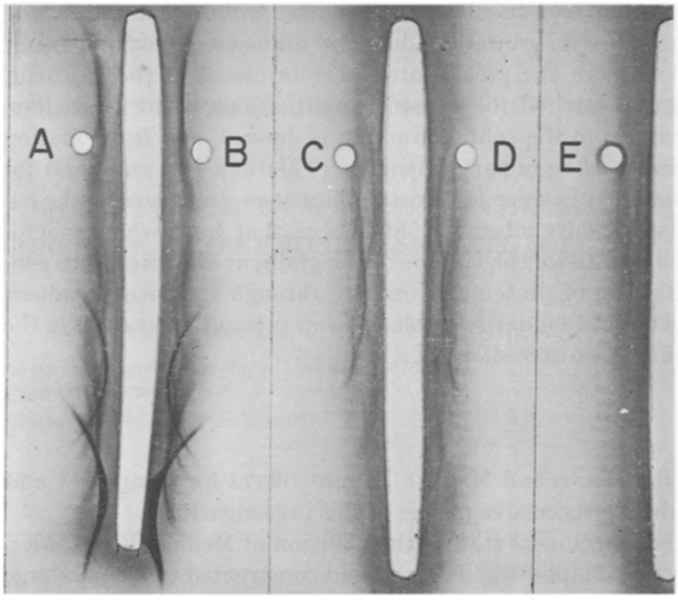

Fig. 5. Immunoelectrophoresis patterns of fractions obtained by solubility chromatography. Pattern A: whole serum; pattern B: soluble fraction from Column 1; pattern C: pooled C'1 from Column 1; pattern D: C'1 Type A from Column 2; pattern E: C'1 Type B from Column 2, examined at one-half the protein concentration of the other samples. The anode is at the bottom.

serum (A); this is not surprising in view of the fact that the pooled C'1 contains only a small fraction (2–3%) of the total serum proteins; because of their low concentration, these proteins are not detected in the pattern for whole serum. The pattern for C'1 Pool B from Column 2 shows three or four arcs, indicating that this preparation is still not homogeneous. The antigen well in this pattern is surrounded by precipitate, which trails off toward the anode; a very faint ring of similar appearance also surrounds the antigen well of pattern C, but not that of pattern D. This precipitate may be due to C'1, which should be virtually insoluble at the ionic strength (0.05) at which the immunoelectrophoretic analysis was carried out.

DISCUSSION

The data presented show that solubility chromatography is a useful method of purification for a protein which is insoluble at low ionic strength. The expected advantages of the method, discussed in the Introduction, appear to have been realized.

There are three ways in which a protein might be applied to the column for solubility chromatography: (i) in precipitated form, preceding the gradient; (ii) in solution, preceding the gradient; and (iii) in solution, after the gradient. The first of these was not suited to the problem at hand, but was studied as an option in the development of solubility chromatography in concentrated ammonium sulfate solutions, which will be considered in the next report. Method (ii) was applied to Column 1; it requires special care in the choice of column volume, for in this case the

salt solution in which the protein was dissolved at the outset will precede the eluting gradient down the column. Unless it is suitably diluted by diffusion during its passage down the column, it will dissolve the precipitated protein ahead of the gradient. In spite of this disadvantage, method (ii) is useful in situations where immediate separation of the precipitate from the soluble fraction is desired, and fractionation of the precipitated proteins is of secondary importance. Method (iii) was used for Column 2; it will be the method of choice for most applications. In this case, the column should be designed so that its internal volume, *i.e.*, that from which macromolecules are excluded, is about twice the volume of the gradient one intends to use; this permits the soluble fraction of proteins to migrate through the entire gradient and to emerge ahead of it, while individual insoluble proteins precipitate in zones in the gradient where their solubilities are exceeded.

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REFERENCES

1 J. PORATH, *Nature*, 196 (1962) 47.
2 R. N. SARGENT AND D. L. GRAHAM, *Anal. Chim. Acta*, 30 (1964) 101.
3 I. H. LEPOW, O. D. RATNOFF AND L. R. LEVY, *J. Exptl. Med.*, 107 (1958) 451.
4 T. BORSOS, H. J. RAPP AND C. T. COOK, *J. Immunol.*, 87 (1961) 330.
5 H. J. MUELLER-EBERHARD AND C. E. BIRO, *J. Exptl. Med.*, 118 (1963) 447.
6 T. BORSOS AND H. J. RAPP, *J. Immunol.*, 91 (1963) 851.
7 R. A. NELSON, JR., J. JENSEN, I. GIGLI AND N. TAMURA, *Immunochem.*, 3 (1966) 111.
8 K. E. FJELLSTRØM, *Acta Pathol. Microbiol. Scand.*, 54 (1962) 439.
9 E. A. KABAT AND M. M. MAYER, *Experimental Immunochemistry*, 2nd Ed., Thomas, Ft. Lauderdale, 1961, p. 162.
10 L. G. HOFFMANN, *Science*, 159 (1968) 322.
11 E. A. KABAT AND M. M. MAYER, *Experimental Immunochemistry*, 2nd Ed., Thomas, Ft. Lauderdale, 1961, p. 200.
12 L. G. HOFFMANN, A. T. MCKENZIE AND M. M. MAYER, *Immunochem.*, 2 (1965) 13.
13 L. G. HOFFMANN AND P. MEIER, *Immunochem.*, 4 (1967) 419.
14 T. BORSOS, personal communication, 1966.
15 E. L. BECKER, *J. Immunol.*, 84 (1960) 299.
16 T. BORSOS, H. J. RAPP AND U. L. WALZ, *J. Immunol.*, 92 (1964) 108.
17 L. ORNSTEIN AND B. J. DAVIS, *Ann. N.Y. Acad. Sci.*, 121 (1964) 321, 404.
18 J. BROOME, *Nature*, 199 (1963) 179.
19 J. J. SCHEIDEGGER, *Intern. Arch. Allergy Appl. Immunol.*, 7 (1955) 103.
20 W. S. STONE, K. AMIRAIAN, C. DUELL AND C. SCHADLER, *Proc. Animal Care Panel*, 11 (1961) 299.
21 J. J. THOMPSON, *Guinea Pig Complement: Functional Studies of Two Active Forms of the First Component*, M. S. Thesis, University of Iowa, 1968.
22 H. R. COLTEN, T. BORBOS AND H. J. RAPP, *J. Immunol.*, 100 (1968) 799.
23 J. J. THOMPSON AND L. G. HOFFMANN, *Federation Proc.*, 27 (1968) 313.
24 K. NAGAKI AND R. M. STROUD, *3rd Complement Workshop*, *J. Immunol.*, 101 (1968) 810.
25 M. M. MAYER, personal communication, 1968.
26 H. R. COLTEN, T. BORSOS AND H. J. RAPP, *J. Immunol.*, 100 (1968) 808.