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# SOLUBILITY CHROMATOGRAPHY OF SERUM PROTEINS

## II. PARTIAL PURIFICATION OF THE SECOND COMPONENT OF GUINEA PIG COMPLEMENT BY SOLUBILITY CHROMATOGRAPHY IN CONCENTRATED AMMONIUM SULFATE SOLUTIONS*

LOUIS G. HOFFMANN AND PATRICIA W. McGIVERN
*Department of Microbiology, University of Iowa, Iowa City, Iowa (U.S.A.)*

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SUMMARY

A method for the chromatographic separation of serum proteins by solubility in concentrated ammonium sulfate solutions has been developed. The fraction of serum proteins which is soluble in 2.20 *M* $(NH_4)_2SO_4$ at pH 6.0 can be resolved into six distinct components detectable by absorbance at 278 or 412 nm. The method is useful for the preliminary purification of the second component of complement from guinea pig serum; recoveries ranged from 23 to 73 %, with a ten-fold increase in specific activity. The effects of a number of variables on separation efficiency are described.

## INTRODUCTION

The preceding report in this series[1] describes a method for chromatographic separation of serum proteins by solubility in buffers of low ionic strength. Here we describe the extension of solubility chromatography to concentrated solutions of ammonium sulfate. The method is applied to the preliminary purification of the second component of guinea pig complement, C'2**, starting with the serum fraction which is soluble at ionic strength, $\mu = 0.020$, pH 5.6.

## MATERIALS AND METHODS

### *Apparatus*

The automatic system for analyzing effluents from chromatography columns, described in the preceding report[1], was used. But recordings of pH could not be obtained because the saturated KCl in the reference electrode junction precipitated with the $(NH_4)_2SO_4$ in the solution being analyzed. (This phenomenon does not

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** The abbreviations used are the same as those given in the preceding report[1].

preclude quick pH measurements on $(NH_4)_2SO_4$ solutions with the usual dipping electrodes, but manifests itself after prolonged contact.) The flow cell used for conductivity measurements had a cell constant of 122.6 $cm^{-1}$. Since the conductivity of concentrated $(NH_4)_2SO_4$ solutions increases with concentration in a non-linear manner, a calibration plot was used to convert conductivity to ammonium sulfate concentration.

*Assay procedures*

The methods used were described previously[1]. The results of C'2 assays were expressed in terms of site-forming units (SFU)[2] per ml; day to day variations in assay sensitivity were estimated by calibration against a standard lot of C'2. This lot, in turn, had been standardized by comparison to two samples of C'2 containing a known number of C'2 units per ml as defined by MAYER AND MILLER[3]; these samples were generously provided by Dr. MANFRED M. MAYER, Department of Microbiology, The Johns Hopkins University School of Medicine.

*Electrophoresis*

The electrophoretic procedures described in the preceding report[1] were followed here, with the exception that samples for disc electrophoresis were diluted to an optical density of 1.5 at 278 nm (1 cm light path).

*Chromatography*

We used a borosilicate glass column, 10.2 cm I.D. × 90 cm long, fitted with removable end pieces and an upward flow adaptor (Glass Engineering Co., Houston, Tex.). The column was coated with dimethyldichlorosilane (Bio-Rad Labs., Richmond, Calif.) and charged with 650 g of Sephadex G-50, particle size 20 to 80 $\mu$. This produced a final bed height of 69 cm under operating conditions. The gel was equilibrated by upward flow with 2.7 $M$ $(NH_4)_2SO_4$, pH 6.0 (Mann Research Labs., Special Enzyme Grade); attempts at equilibration by downward flow were complicated by convective disturbances due to density differences between concentrated $(NH_4)_2SO_4$ solutions. Between successive runs, the bed of this column was completely stirred up to prevent excessive packing, which reduces the flow rate; part of the gel was temporarily removed from the column to accomplish this. The operation was usually performed before equilibration. The exclusion and total volumes of the column were determined with Blue Dextran (Pharmacia Fine Chemicals, Piscataway, N.J.) and $(NH_4)_2SO_4$, respectively; the difference between the two volumes was 4.1 l, and was assumed to remain constant in spite of small variations in total bed volume due to differences in packing from run to run.

Chromatographic separations were performed by downward flow. First, a non-linear 1.8 l gradient of $(NH_4)_2SO_4$ concentrations decreasing from 2.70 $M$ to 1.80 $M$ was applied. Each chamber of the gradient former contained 200 ml of the solution indicated in Table I; all solutions were adjusted to pH 6.0.

The protein fraction soluble at pH 5.6, ionic strength $\mu = 0.020$, was obtained from 200 ml of guinea pig serum by solubility chromatography[1]. The total amount of protein to be applied to the Sephadex column was reduced by a preliminary $(NH_4)_2SO_4$ precipitation process. Solid $\varepsilon$-amino-$n$-caproic acid (EACA, Calbiochem, Los Angeles, Calif.) was added to the protein solution to a concentration of 0.1 $M$ to inhibit serum

TABLE I

AMMONIUM SULFATE GRADIENT SOLUTIONS

| | *Chamber* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *1* | *2* | *3* | *4* | *5* | *6* | *7* | *8* | *9* |
| $[(NH_4)_2SO_4]$, *M* | 2.70 | 2.65 | 2.55 | 2.50 | 2.45 | 2.40 | 2.35 | 2.20 | 1.80 |
| [EACA[a]], *M* | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 |

[a] ε-Amino-*n*-caproic acid.

proteases; then, solid $(NH_4)_2SO_4$ was added to a concentration of 2.20 *M*, and the pH was adjusted to 6.0. The amount of $(NH_4)_2SO_4$ required was determined on the basis of density measurements on a series of $(NH_4)_2SO_4$ solutions. All additions were performed in an ice-water bath. The solution was stirred at 0° for another 30 min and then centrifuged at —5° for 20 min at 10,000 × *g*. The resulting precipitate generally contained less than 10% of the C'2 activity present in the starting material. The proteins in the supernatant fluid were precipitated by addition of further $(NH_4)_2SO_4$ to a concentration of 3.50 *M*. The mixture was adjusted to pH 6.0 and stirred for 30 min at 0°, and then centrifuged at —5° for 1 h at 10,000 × *g*. The resulting supernatant fluid was checked for protein by measuring the optical density (O.D.) at 278 nm of a 1:2 dilution in $H_2O$; the O.D. values obtained were less than 0.04. The precipitate was resuspended in a minimal amount of 3.50 *M* $(NH_4)_2SO_4$, pH 6.0.

These precipitation procedures were always carried out immediately after the protein emerged from the pH 5.6, $\mu = 0.020$ solubility chromatography column. The precipitated crude C'2 fraction was stored at —65° until use; under these conditions, there was no loss of activity for several months.

For application to the column, the suspension of precipitate was carefully thawed, redissolved in an equal volume of ice-cold 0.2 *M* EACA, and centrifuged at —5° for 30 min at 10,000 × *g*. The supernatant fluid was pumped into the column immediately after the gradient, followed by 1.80 *M* $(NH_4)_2SO_4$, pH 6.0.

Flow rates close to, but not exceeding, 200 ml/h were maintained; higher flow rates led to artefacts. The first 2.7 l of effluent contained no protein and were collected into a graduate cylinder; then, 20 ml fractions were collected into 2 ml of 3.50 *M* $(NH_4)_2SO_4$, pH 6.0, per tube. This procedure was designed to minimize degradation of the C'2 in the collected fractions by precipitating most of the protein.

After assay, the relevant fractions were pooled and the conductivity of the solution was measured. The $(NH_4)_2SO_4$ concentration corresponding to this conductivity was determined from a calibration chart, and the amount of solid $(NH_4)_2SO_4$ to be added to the solution to adjust its concentration to 3.50 *M* was calculated from the density data referred to earlier. The addition of this $(NH_4)_2SO_4$ was preceded by sufficient solid EACA to increase its concentration by 0.10 *M*. The pH was adjusted to 6.0 and the mixture was stirred at 0° for 30 min, followed by centrifugation at —5° for 30 min at 10,000 × *g*. The supernatant fluid was checked for protein concentration as above, and the precipitate was resuspended in a minimal volume of 3.50 *M* $(NH_4)_2SO_4$, pH 6.0, and stored at —65°.

The normal running time for this column is 48 h from the time of application of the protein.

RESULTS

A typical chromatogram is shown in Fig. 1. Four peaks—three major and one minor—can be identified by O.D. at 278 nm. The O.D. at 412 nm, for proteins absorbing in the Soret band, reveals two additional peaks which do not coincide with any of the 278 nm peaks. The O.D. curve at 360 nm shows that both 412 nm peaks represent absorbance rather than just turbidity, for in that case the O.D. at 360 nm would exceed that at 412 nm. The small spike in O.D. at 278 nm, centered over fraction 137, is seen in some, but not all, chromatograms.

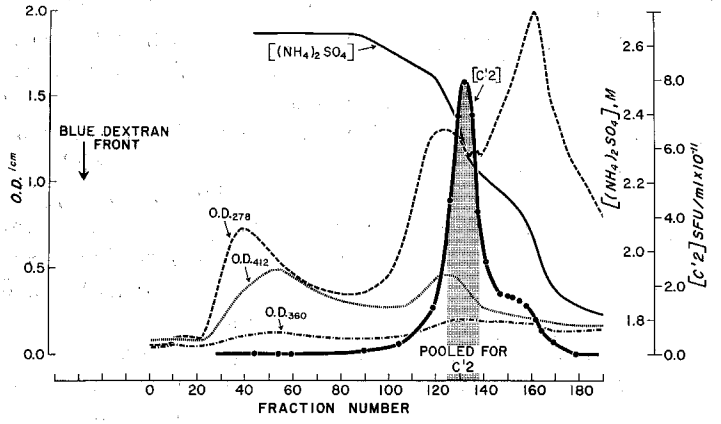


Fig. 1. Solubility chromatography of the serum protein fraction soluble in 2.20 $M$ $(NH_4)_2SO_4$, in concentrated $(NH_4)_2SO_4$ solutions. Fractions: 20 ml. Calibration of assay system shows $1.84 \times 10^8$ SFU/unit of C'2.

None of the proteins emerges in the exclusion volume, even though several peaks occur in a region of the chromatogram where the $(NH_4)_2SO_4$ concentration is a constant 2.7 $M$. (The difference between 2.7 $M$ and the indicated value probably arises from a combination of instrument and calibration errors.) This indicated that none of these proteins is completely soluble in 2.7 $M$ $(NH_4)_2SO_4$, and that they are retarded on the column to different degrees depending on their solubilities in this medium.

The C'2 emerges in a narrow zone with some tailing; the amount of tailing is usually less than shown here. No peak in O.D. at 278 nm is associated with the C'2 maximum, but this is not surprising because of the crude nature of the starting material. The elution position of the C'2 is highly reproducible: In four of the six runs for which results are presented in Table II, the maximum in C'2 concentration occurred at 2.28 $M$ $(NH_4)_2SO_4$; in the remaining two runs it occurred at 2.19 $M$ and 2.37 $M$ $(NH_4)_2SO_4$, but the position of the C'2 band relative to the $O.D._{278}$ pattern was unchanged.

The recoveries and degrees of purification for six representative runs are presented in Table II. Purification was calculated as increase in specific activity on the basis of measurements of the O.D. at 278 nm of samples diluted in $H_2O$ so as to dissolve any precipitate present. The recoveries vary from run to run; this probably

TABLE II

RESULTS OF SOLUBILITY CHROMATOGRAPHY IN CONCENTRATED $(NH_4)_2SO_4$

| *Run no.* | *2.2 to 3.5 M $(NH_4)_2SO_4$ precipitate* | | *Pooled fractions* | | | |
|---|---|---|---|---|---|---|
| | | | *Based on input* | | *Over-all* | |
| | *% Yield*[a] | *Purification*[a] | *% Yield* | *Purification* | *% Yield* | *Purification* |
| XXXIII-6 | 63[b] | 1.6 × | 92 | 5.1 × | 73 | 11.5 × |
| XXXIV-3 | 73 | 2.0 × | 26 | 3.7 × | 28 | 10.9 × |
| XXXV-3 | 61 | 1.7 × | 41 | 4.5 × | 44 | 12.1 × |
| XXXVI-3[c] | 68 | 1.7 × | 38 | 3.1 × | 35 | 7.2 × |
| XXXVII-3 | 40 | 3.2 × | 73 | 7.5 × | 23 | 10.5 × |
| XXXVIII-3 | 79 | 2.0 × | 46 | 5.4 × | 34 | 10.4 × |

[a] Based on whole serum; these figures are derived from assays of samples of resuspended precipitate, and are thus less reliable than usual.

[b] After correction for 45.9% mechanical loss on 1st column[1].

[c] A different gradient was used in this run; *cf.* text.

reflects the extreme lability of the protein being purified. An example of this lability is given by NELSON *et al.*[4], who state that ion exchange chromatography of the serum protein fraction soluble at pH 7.5, $\mu = 0.04$, on diethylaminoethyl cellulose leads to "low" C'2 recovery. The exceptionally low yield of C'2 in run XXXVII-3 can probably be ascribed to a technical error which occurred in the initial precipitation procedure. In run XXXVI-3, a different gradient from the one described in Table I was used in an attempt to improve resolution; this gradient was shallower in the region where C'2 elutes. Evidently, this results in less purification than is obtained with the gradient shown in Table I.

The disc electrophoresis patterns in Fig. 2 give a qualitative picture of the progress of purification through the procedures described. By comparison with pattern A (proteins soluble at pH 5.6, $\mu = 0.020$), pattern B shows that precipitation with 2.20 *M* $(NH_4)_2SO_4$ removes most of the $\beta$- and $\gamma$-globulins, and results in a concurrent enrichment in the albumin and $\alpha$-globulin regions. Solubility chromatography in $(NH_4)_2SO_4$ (pattern C) eliminates the remaining protein of slow $\gamma$ mobility at 0.5 cm and markedly alters the composition of the $\alpha$-globulin region (2.5 to 3.5 cm), without appreciably affecting the amounts of albumin and of residual $\beta$-globulin (2.0 cm) present.

Examination of the immunoelectrophoresis patterns in Fig. 3 leads to essentially the same conclusions. Pattern C (for the 2.20 *M* $(NH_4)_2SO_4$ supernatant fluid) is remarkable for the absence of an arc corresponding to the prominent slow $\gamma$-globulin seen in the corresponding disc electrophoresis pattern; either the antiserum used contains no antibody against this protein, or the molecular weight of this constituent is high enough to cause an appreciable reduction in its electrophoretic mobility in 7.5% polyacrylamide gel because of exclusion effects.

## DISCUSSION

The purpose of the present study was to develop a new class of chromatographic procedures for proteins, based on differences in solubility, which would be particularly

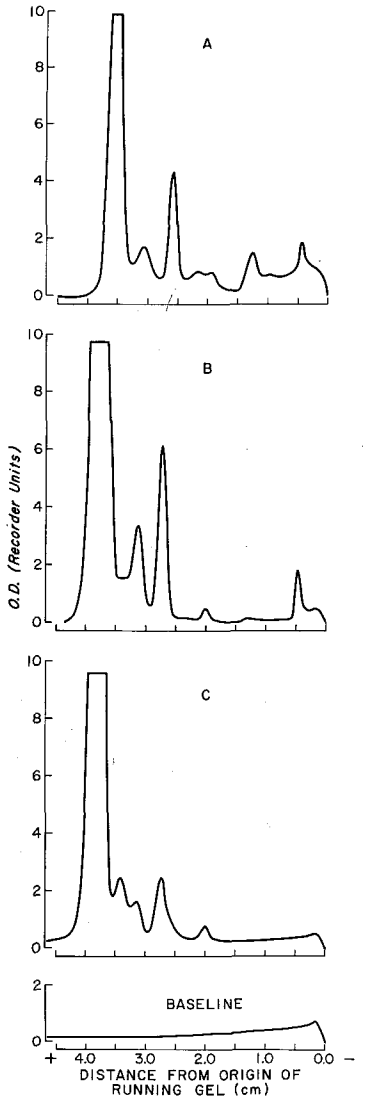


Fig. 2. Disc electrophoresis patterns of partially purified C′2 preparations. Pattern A: serum proteins soluble at pH 5.6, $\mu$ = 0.020 (starting material). Pattern B: fraction soluble in 2.20 $M$ $(NH_4)_2SO_4$, pH 6.0. Pattern C: concentrated C′2 fraction. Baseline: pattern obtained from a gel without protein.

suited for application to proteins that are labile in solution, and to situations where it is desirable to process substantial quantities of protein. The preliminary purification of C′2 from guinea pig serum, in which it is present in very small amounts, is a problem that presents both of these difficulties.

The first chromatographic procedure for the preparation of partially purified C′2 was developed by BORSOS *et al.*[5] in 1961. The largest amount of serum that can be handled conveniently by this method is 40 ml, and the required time is one day. The

product has the great advantage of being stable. The increase in specific activity over serum is less than 10-fold, but recoveries have been rather variable in our hands, indicating that the method is unduly sensitive to minor variations in technique. C'2 preparations made by this method are free of C'1 and C'4 (ref. 5), but some of the other C' components may be present in substantial amounts.

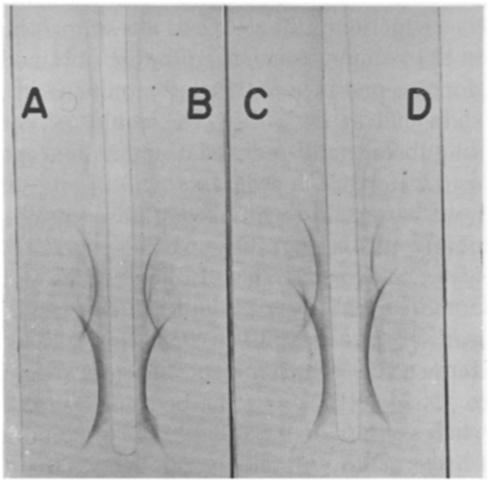


Fig. 3. Immunoelectrophoresis patterns of serum and fractions. Pattern A: whole guinea pig serum. Pattern B: serum proteins soluble at pH 5.6, $\mu = 0.020$. Pattern C: fraction soluble in 2.20 $M$ $(NH_4)_2SO_4$, pH 6.0. Pattern D: concentrated C'2 fraction.

NELSON *et al.*[4] have described a method for purification of C'2 by two successive steps of ion exchange chromatography on carboxymethyl cellulose at pH 5.0 and 5.5, respectively. This procedure requires several days, and the amount of serum handled in one preparation is 30 ml. The product is free of other C' components, but the small amounts of material obtained have not permitted application of criteria of chemical purity. In the only run for which NELSON *et al.* supply information on recovery, it appears to have been about 75%; inactivation was apparently circumvented by working at rather high dilutions.

Recently, POLLEY AND MUELLER-EBERHARD[6] have reported the isolation of a highly purified fraction with C'2 activity from human serum by a sequence of steps consisting of ion exchange chromatography on carboxymethyl cellulose, electrophoresis on Pevikon gel, and chromatography on hydroxyapatite. In their procedure, 700–800 ml of serum are handled at one time. In the two runs for which their report provides details, recovery in the first step was 29 and 32 %, with an 8- to 18-fold increase in specific activity. In order to achieve these results, POLLEY AND MUELLER-EBERHARD found it necessary to treat their starting material with diisopropyl fluorophosphate (DFP) to inactivate serum proteases. Their report provides no information on contamination with other C' components.

In our study, we have developed a method by which 200 ml of serum can be processed in a total of three days of column operation (not counting preparation of solutions, etc.) to yield a partially purified product, usually in 28 to 44 % yield at 10- to 12-fold purification. The C'2 was protected from enzymatic inactivation by the

presence of 0.1 *M* EACA; it would be impossible to use this inhibitor at this concentration in ion exchange chromatography, since such a high level of zwitterions would interfere with the ion exchange process. The ability to use this substance, rather than the highly toxic and volatile DFP, to protect the C'2 constitutes one of the advantages of this method. We have not tried to process more than 200 ml of serum at a time, but there appears to be no reason why larger amounts could not be handled on the same column; however, one might anticipate some reduction in flow rate as a result of the presence of larger amounts of precipitate on the column. Since the product obtained is still far from pure, we have not looked for the presence of other C' components; on the basis of the $(NH_4)_2SO_4$ solubility data of NELSON *et al.*[4], we would expect most of the C' components to be present in substantially reduced amounts, except for C'9. Efforts at further purification of this fraction are in progress.

In our efforts to develop this method, we have made a number of observations that may be useful in the application of solubility chromatography to other problems.

We have compared two types of gel, *viz.*, polyacrylamide (Bio-Gel P-10) and dextran (Sephadex G-50) gels. Although some conditions may indicate the use of polyacrylamide[1], Sephadex G-50 would appear to be the medium of choice for work at high ionic strength and consequent low water activity, which affects the water regain of the gel. On changing from 1 *M* NaCl to 3.5 *M* $(NH_4)_2SO_4$, the bed volume of a Bio-Gel P-10 column dropped by 50%, whereas that of a Sephadex G-50 column decreased by 30%; the latter gel showed little or no volume change below 2.7 *M* $(NH_4)_2SO_4$, the range in which fractionation took place in this study. We have found that the particle size of the gel is of some importance; as in other applications of gel filtration, the sharpest separation is attained with the smallest particle size. No other gels were evaluated.

Since separation is occurring on the basis of precipitation and dissolution, which are relatively slow processes, one might expect the flow rate to play a crucial role. This has turned out to be the case; we found that flow rates above 2.5 cm/h led to extensive tailing, indicating that equilibrium was not being attained. For this reason, the use of a coarse gel particle size provides no advantage over the fine particle size, since the higher flow rate afforded thereby would be detrimental to separation.

In this study, we have examined two methods of applying the protein sample to the column. In addition to the method described, we tried placing the protein on the gel in the form of a slurry of precipitate before applying the gradient, hoping that the gradient would selectively dissolve the precipitate as it passed through; this would have avoided altogether the need to handle the protein in solution. This method of application did not yield satisfactory results because the liquid flowing through the precipitate created channels, leaving behind islands of undissolved protein. The method described is therefore the method of choice.

The slope of the $(NH_4)_2SO_4$ concentration gradient has a predictable effect. In run XXXVI-3 (Table II) a gradient was used which was shallower in the region of C'2 elution than the one described in Table I. In this run the C'2 was eluted at nearly the same position, but in a zone that was approximately twice as broad as in the other runs, with a slight loss in purification efficiency. Steeper gradients were not tried, but stepwise elution appears undesirable—we have found that a 0.5 *M* reduction in $(NH_4)_2SO_4$ concentration in a single step produces marked tailing in both protein and $(NH_4)_2SO_4$ concentrations, extending over more than a liter, in the effluent. This

artefact may be due to a failure to attain equilibrium across a sharp decrease in $(NH_4)_2SO_4$ concentration; however, this argument does not explain the tailing with respect to $(NH_4)_2SO_4$ concentration.

Fig. 1 shows that some proteins have migrated through the gradient and penetrated fairly deeply into the region on the column where the $(NH_4)_2SO_4$ concentration is 2.7 *M*, without emerging with the void volume. This observation can be explained on the assumption that the proteins in question are partially soluble in 2.7 *M* $(NH_4)_2SO_4$; if the column is equilibrated with 3.5 *M* $(NH_4)_2SO_4$ before application of the sample, all the protein emerges in the gradient. In the case of C'2 the concentration of $(NH_4)_2SO_4$ with which the column is equilibrated has a marked effect on migration. In spite of the fact that under the conditions described, the C'2 emerges in the gradient at 2.28 *M* $(NH_4)_2SO_4$, it will elute only slightly behind the void volume of a column equilibrated with 2.5 *M* $(NH_4)_2SO_4$. On a column equilibrated with 2.6 *M* $(NH_4)_2SO_4$, part of the C'2 emerges ahead of the gradient, the rest early in the gradient. In either case, the C'2 zone is very broad, extending over a liter or more of effluent, and purification is poor. These observations may be summarized by suggesting that in a critical range of $(NH_4)_2SO_4$ concentrations, it is possible to achieve a true counter-current process, but that a shallow gradient will produce the best results.

It seems likely that the results described in this report for solubility chromatography in concentrated $(NH_4)_2SO_4$ solutions can be generalized to other precipitants, including organic solvents; the latter could be used in conjunction with the lipophilic Sephadex LH-20. We have explored the applicability of solubility chromatography in ethanol on Sephadex LH-20 as a step in the purification of C'2, but have been discouraged from pursuing it further by the occurrence of artefacts and by very poor recoveries.

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