1986

A Preliminary Study on the Separation of Bovine Plasma Albumin-Copper Ion Dimer and Trimer Reaction Products by Ion-Exchange Chromatography

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A PRELIMINARY STUDY ON THE SEPARATION OF BOVINE PLASMA ALBUMIN-COPPER ION DIMER AND TRIMER REACTION PRODUCTS BY ION-EXCHANGE CHROMATOGRAPHY

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Submitted in partial fulfillment of the

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Abstract

When bovine plasma albumin is in the presence of copper metal ions at pH values below the isoelectric point, a dimerization reaction takes place between the protein molecules. The kinetics of this reaction have been previously studied by using light scattering techniques. These techniques involve monitoring the amount of light scattered as the dimerization reaction proceeds. As the amount of dimer present increases, more light is scattered due to increased molecular size in the solution. Accurate calculations of the rate of this reaction depend upon knowing the ratio of dimer to monomer concentrations at any given time. Unfortunately, evidence has indicated that a small amount of trimer is also formed towards the end of this reaction. This complicates determination of the dimer concentration to the extent that definite conclusions cannot be drawn concerning reaction rates.

This study addresses the possibility of using ion-exchange chromatography as a means of separating the BPA monomer, dimer, and trimer products so that their respective amounts can be more accurately determined. Hence, the data obtained will be a better reflection of the kinetics actually involved in this reaction.

Introduction

Some of the interesting characteristics of various reactions are their kinetics. This involves studying how the rate at which a chemical reaction takes place can be affected by varying the conditions under which it occurs. Information of this type helps in the determination of what allows a compound to react as it does.

The compound of interest in this study is bovine plasma albumin (BPA), a protein found in cow's blood. Proteins are large molecules composed of amino acids which play important roles in biological systems. For example, BPA and its human counterpart are used to transport particular substances throughout the body and help to maintain osmotic pressure between tissue fluid and the blood. When BPA and copper metal ions --Cu(II)-- are combined they react to produce a dimer (two proteins linked together) and a small amount of trimer (three linked proteins). One view proposed by Everett (1968) is that copper serves as a catalyst in the formation of disulfide bonds between BPA molecules. A source of research in this area, also described by Everett (1968), has been how the kinetics of this reaction are affected by various conditions such as pH, ionic strength, and Cu(II) concentration.

The above mentioned study involves analyzing the amount of light which is scattered by these proteins in solution. As the dimerization reaction progresses, the solution becomes more turbid due to the increasing amount of dimer and more light is scattered. Therefore, the increase in light scattering observed over a period of time is proportional to the amount of dimer produced during that time. Determining the ratio of dimer concentration to unreacted monomer

concentration in the solution as the reaction proceeds is crucial to studying the kinetics involved. Unfortunately, the trimer which is produced complicates the determination of this ratio so that definite conclusions cannot be drawn.

The method needed to allow conclusions to be drawn concerning the kinetics of this reaction involves taking representative samples from the BPA-Cu(II) mixture over specified intervals of time and stopping the reaction in each sample with EDTA. The EDTA molecules stop the reaction by binding with the Cu(II) so that it is no longer free to react with BPA. The sample is then run through a chromatography column which separates the dimer from the unreacted BPA monomer. Finally the amounts of monomer and dimer can be determined. Obtaining accurate results relies on realizing that the trimer produced by this reaction must also be separated from the solution. Only after this separation has been perfected can the reaction kinetics be accurately studied.

Previous studies of column chromatography conducted by Everett focused on molecular exclusion techniques where molecular size was the primary means of separation. Satisfactory resolution of monomer and dimer was obtained with this method. However, the trimer consistently appeared as a shoulder on the dimer peak and did not lend itself to

clear separation. Another hindrance to molecular exclusion chromatography was the large volume of sample required for adequate separation of these reaction products.

This study will focus on the possible use of ion-exchange chromatography as a satisfactory means of separating the BPA-Cu(II) reaction products. Since this reaction is carried out under acidic conditions (pH 3.0), well below the isoelectric point of BPA, a cation exchange resin is required. Various solution parameters such as pH and ionic strength will then be altered in the eluting solutions to optimize separation.

Materials and Methods

The bovine plasma albumin used in this project was obtained as the crystallized protein from United States Biochemical Corporation. All inorganic reagents were analyzed reagent grade. SP-Sephadex C-50 was used as supplied by Pharmacia Fine Chemicals.

Conductivity measurements were performed with an Industrial Instruments Model RC 16B2 Conductivity Bridge. The pH determinations were made using either a Beckman Zeromatic pH Meter or an Orion Model 231 Digital pH Meter. Both meters were calibrated at two points with buffer solutions of pH 4.00 and pH 7.00.

Dialysis membranes were made from cellulose dialysis tubing purchased from Spectrum Medical Industries. The tubing used in the dialysis procedures was treated with a hot solution of sodium bicarbonate and then washed repeatedly with distilled water as described by Everett (1963). All tubing was kept refrigerated until its later use.

Preparation of SP-Sephadex C-50 Column

Approximately 8 grams of SP-Sephadex C-50 were suspended in 500 ml of distilled water and allowed to sit overnight until swelling was complete. The swelled Sephadex was initially rinsed twice with 500 ml of 0.1 N NaCl. It was then suspended in 500 ml of 0.5 N HCL, washed with distilled water, suspended in 500 ml of o.5 N NaOH, and washed with distilled water until the conductivity of the eluate was within 100 mhos of distilled water. This procedure described by the manufacturers was followed to remove any heavy metal impurities which may have been present. The Sephadex was then rinsed with "pouring buffer" (0.02 M Na acetate - 0.18 M NaCl, pH 4.30) until the conductivity of the eluate was within 1.0% of the "pouring buffer". Conductivity measurements were used as a means of ensuring that equilibrium had been reached.

Preparation and Dimerization of BPA Solution

Ten milliliters of a 1.25% BPA solution were made using crystallized protein. In order to carry out light-scattering studies on bovine plasma albumin at pH values below approximately 4.0, Everett (1968) states that the fatty acid bound to the protein must first be removed by the method of Chen (1967). This involved the use of Norite charcoal previously acid washed to remove impurities and then washed with distilled water and dried. To 10 ml of the 1.25% BPA solution were added 0.5 grams of charcoal. This solution was then adjusted to pH 2.75 with 0.1 M HCL and allowed to stand overnight in the refrigerator. The charcoal was removed by centrifugation at $4^{o}C$ and filtering through 0.45 millipore membrane filters.

The defatted BPA solution was then brought to pH 3.00 with 0.1 M HCL and an ionic strength of 0.1 with 0.1 M NaCl. To induce dimerization of BPA, 0.1 M $CuCl_2$ was added to the solution to give a final concentration of 1.0×10^{-3} Cu(II). After sitting for two hours, the solution was dialyzed overnight in the refrigerator against 600 ml of "pouring buffer" to remove the Cu(II). The dimerized BPA solution was then stored in the refrigerator.

Ion-Exchange Chromatography

The chromatography procedures used in this study were generally modifications of techniques described by Hagenmaier (1971). The columns were prepared as previously described and equilibrated between trials with "pouring buffer". Once equilibrium had been reached, the dimerized BPA solution was applied to the columns. A salt gradient was used for eluting purposes where the ionic strength was varied by linearly increasing the NaCl concentration and keeping the ratio of sodium acetate to acetic acid concentration constant. Hagenmaier (1971) states that buffers should be made 0.02 M in sodium acetate as higher concentrations of acetate yield much poorer resolution of the peaks. In this work it was found that the pH had to be varied by changing the acetic acid concentration in order to elute the protein and give reasonably sharp peaks.

All columns used in this project were operated under an ascending eluant flow. This method was found to produce a more constant flow rate than the descending column alternative. The size of fractions varied but all were collected by a Buchler Fractomat fraction collector. Columns were maintained at 4-5^{o}C and fractions stored in the refrigerator. Percent transmittance measurements at 280 nm were performed with a Varian Series 634 spectrophotometer on

all fractions collected using "pouring buffer" as a blank. These values were converted to absorbance and then plotted on a graph of absorbance versus tube number.

Results

The initial step in the separation of the BPA components was to determine the necessary conditions of pH and NaCl concentration in eluting solutions to bring these proteins off the column. In the first trial a 15 x 1.5 cm column was equilibrated with pouring buffer which was 0.18 M NaCl - 0.02 M Na acetate, pH 4.3. A 27.5 mg sample of dimerized BPA solution (preparation described earlier) was then loaded onto the column with pouring buffer and collected in 5 ml fractions by eluting with a series of solutions. These solutions were all 0.02 M in Na acetate and of pH 4.3, but they differed in NaCl concentrations. As the NaCl concentration was increased to 0.43 M, the elution profile produced was very sporadic and inconsistent. After 0.43 M NaCl was reached the profile seemed to be better behaved but was still too broad for satisfactory results to be obtained. This indicated that varying the NaCl concentration alone would not be sufficient to elute the BPA components from the column as was hoped. As a result a cleaning solution (0.64 M NaCl - 0.02 Na acetate, pH 5.6) was prepared in which both

the pH and NaCl concentration were increased. It was found that this solution eluted any remaining components from the column.

For the next trial a continuous gradient was used between pouring buffer (0.18 M NaCl - 0.02 M Na acetate, pH 4.3) and the cleaning solution (0.64 M NaCl - 0.02 Na acetate, pH 5.6) to see if the BPA components could be eluted as individual peaks. Figure 1 shows that a single peak centered at fraction 28 was produced by this method. Also indicated along this peak is a shoulder starting at fraction 31. This is probably the result of initial monomer and dimer separation. The addition of more cleaning buffer to the column resulted in the elution of unidentified components in fractions 50-84. No more components were detected after fraction 85.

It should be noted that the column was left alone during the gradient elution for approximately two hours. Upon returning it was found that the resin bed had shrunk as a result of the increasing ionic strength of eluting solutions. A mixing chamber of approximately 2 cm in height formed above the column. It is possible that some of the BPA components could have escaped into this chamber and evaded elution until the latter stages.

A subsequent trial in which the gradient steepness was decreased in both pH and NaCl concentration produced the profile seen in Figure 2. The components are seen to be better resolved in this profile than before with two peaks being indicated, one centered at fraction 53 and the other at fraction 66. Additional elution once again produced unidentifiable components from fractions 90-152. On this and all later trials the column was compressed as it reduced in size so that a mixing chamber was not allowed to form.

Figure 3 shows the elution profile for a sample of crystallized BPA which had not been defatted or dimerized. The same elution solutions were used as for Figure 2. Since the columns used to fractionate these two samples varied slightly in height and since the crystallized BPA was added at a somewhat earlier time, there is not an exact fraction number to peak height correspondence between the two profiles. Still, the similarity between the two elution profiles indicates that the peaks in Figures 1 and 2 are the result of BPA components. It should be noted that the later unidentified components are also present in the crystallized BPA. This indicates the presence of impurities from the original sample have been carried throughout the dimerization procedure.

A final trial was run on dimerized BPA, this time with a lower initial NaCl concentration. All other factors were the same as for Figure 2. Figure 4 shows the results of this trial. The two BPA component peaks centered at fractions 51 and 66 are sharper and better defined than previously. Also fractions 80-87 appear to be a shoulder representing a little trimer separation. It should be noted that the peaks are higher than before, and that the later unidentified components are not as prominent.

Discussion

An accurate study of BPA-Cu(II) reaction kinetics depends upon determining (as accurately as possible) the concentrations of the products formed. Since monomer, dimer, and trimer all result from this reaction, these components must be separated before their concentrations can be determined. Although satisfactory for monomer and dimer separations, molecular exclusion chromatography requires relatively large volumes and leaves the trimer component unresolved. This report has studied the possible use of ion-exchange chromatography in place of molecular exclusion techniques for these separations.

The first thought was that NaCl concentration changes would be enough to elute the BPA components from the column. When this failed to work, a pH gradient was also utilized. Unfortunately, the gradient was too steep and resulted in poor resolution. It also became apparent that the column was being subjected to drastic ionic strength changes and was shrinking to compensate for the imbalance between resin particles and eluting solutions. This meant that the column had to be compressed periodically to avoid the formation of a mixing chamber.

By adjusting the pH and NaCl concentration gradients, it was found that the monomer and dimer components could be separated into two identifiable peaks with increasing resolution. On the final trial it is believed that the shoulder on the dimer peak is indicative of trimer component.

An additional consideration is the identification of the components once they have been eluted from the column. On certain trials it appeared that the BPA solution was precipitating after being loaded onto the column. If this was indeed the case, then it is probable that the later components are simply redissolved BPA components coming off after the bulk has been eluted. This could also account for the higher peaks observed in Figure 4 where there were fewer late components present. All of the BPA came off in the gradient elution instead of after it.

Although the need for more study is obvious, this report has shown that ion-exchange chromatography is a possible technique for the separation of BPA-Cu(II) reaction products. Additional research on pH and NaCl gradients should yield even better results with monomer, dimer, and trimer separations. Identification of these components with electrophoresis or other methods would be necessary for satisfactory conclusions to be drawn.

Figure 1

SP-Sephadex C-50 chromatography of charcoal-defatted, dimerized BPA. The resin bed was 15 x 1.5 cm in size and equilibrated with pouring buffer (0.18 M NaCl - 0.02 M Na acetate, pH 4.3). A 25.0 mg BPA sample was loaded onto the column with pouring buffer and collected in 5 ml fractions by eluting with the following solutions (all 0.02 M Na acetate):

1. continuous gradient:
   200 ml of 0.18 M NaCl, pH 4.3
   200 ml of 0.64 M NaCl, pH 5.6
2. 100 ml of 0.64 M NaCl, pH 5.6

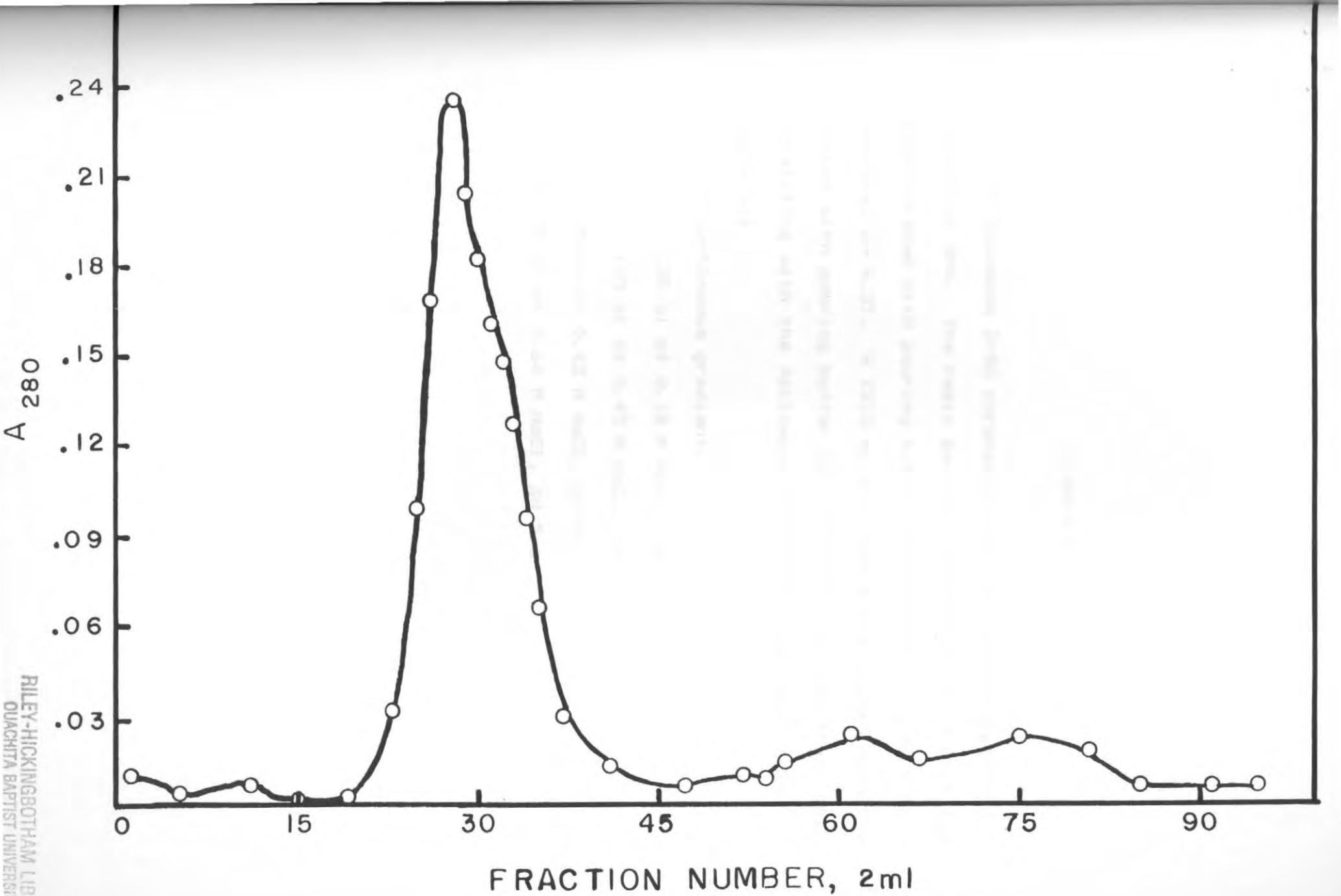


Figure 2

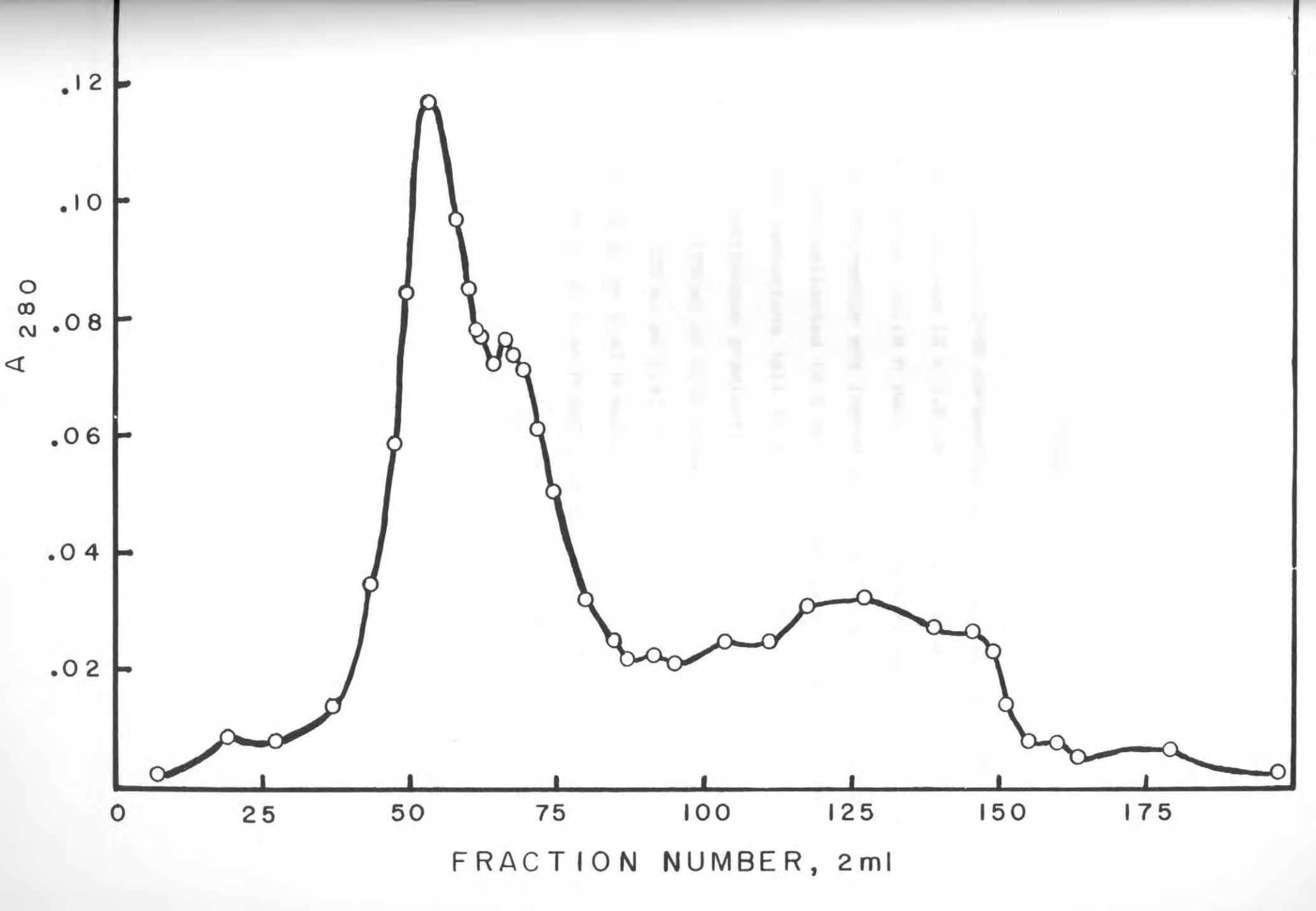
SP-Sephadex C-50 chromatography of charcoal-defatted, dimerized BPA. The resin bed was 12.5 x 1.5 cm in size and equilibrated with pouring buffer (0.18 M NaCl - 0.02 M Na acetate, pH 4.3). A 12.5 mg BPA sample was loaded onto the column with pouring buffer and collected in 2 ml fractions by eluting with the following solutions (all 0.02 M Na acetate):

1. continuous gradient:

   125 ml of 0.18 M NaCl, pH 4.3

   125 ml of 0.43 M NaCl, pH 5.0

2. 75 ml of 0.43 M NaCl, pH 5.0
3. 75 ml of 0.64 M NaCl, pH 5.6


.12
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.08
.06
.04
.02
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25
50
75
100
125
150
175
FRACTION NUMBER, 2ml

Figure 3

SP-Sephadex C-50 chromatography of crystallized BPA. The resin bed was 13 x 1.5 cm in size and equilibrated with pouring buffer (0.18 M NaCl - 0.02 M Na acetate, pH 4.3). A 12.5 mg BPA sample was loaded onto the column with pouring buffer and collected in 2 ml fractions by eluting with the following solutions (all 0.02 M Na acetate):

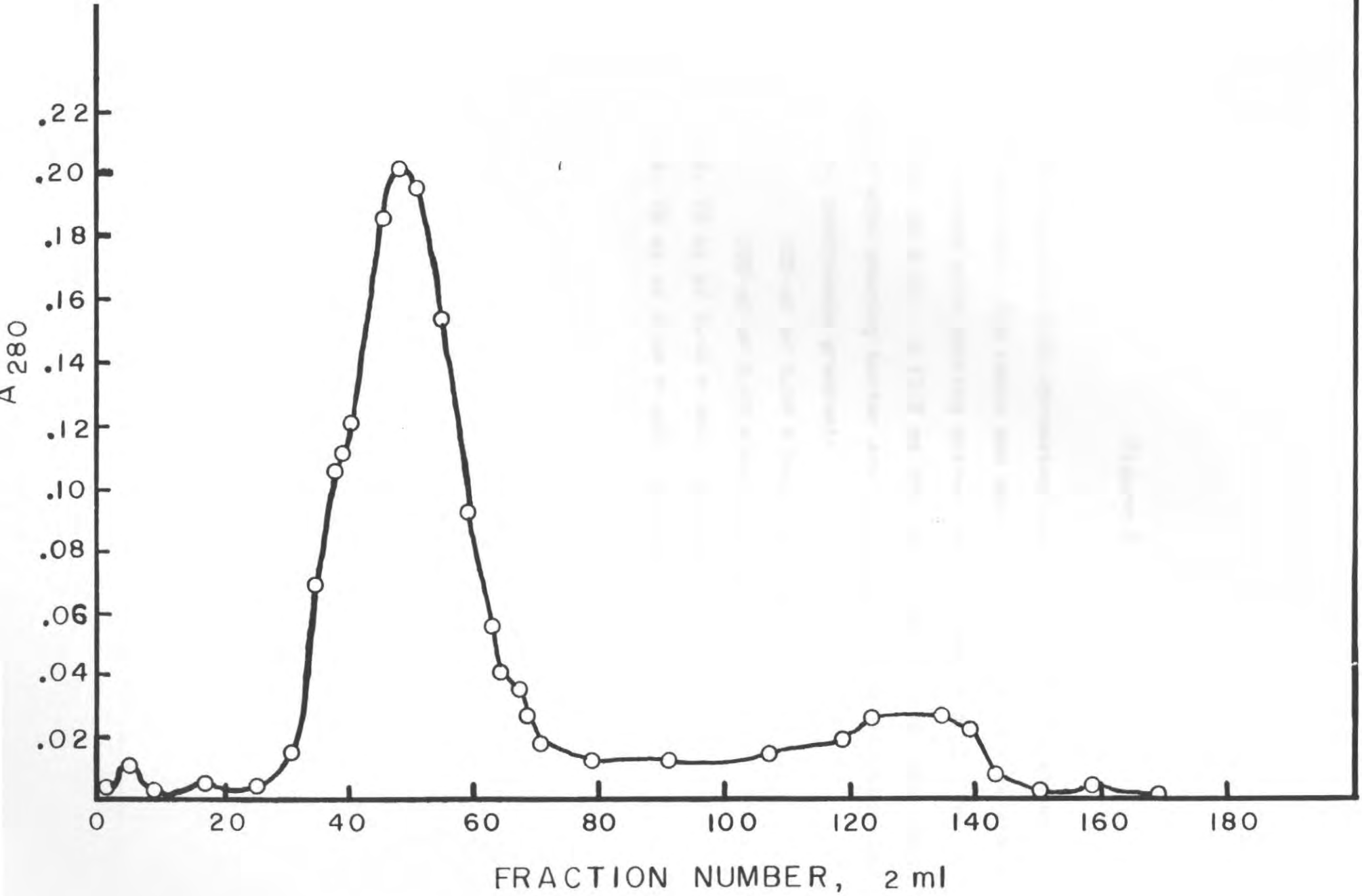
1. continuous gradient:

   125 ml of 0.18 M Nacl, pH 4.3

   125 ml of 0.43 M NaCl, pH 5.0

2. 75 ml of 0.43 M NaCl, pH 5.0

3. 75 ml of 0.64 M NaCl, pH 5.6


.22
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A 280
0
20
40
60
80
100
120
140
160
180
FRACTION NUMBER, 2 ml

Figure 4

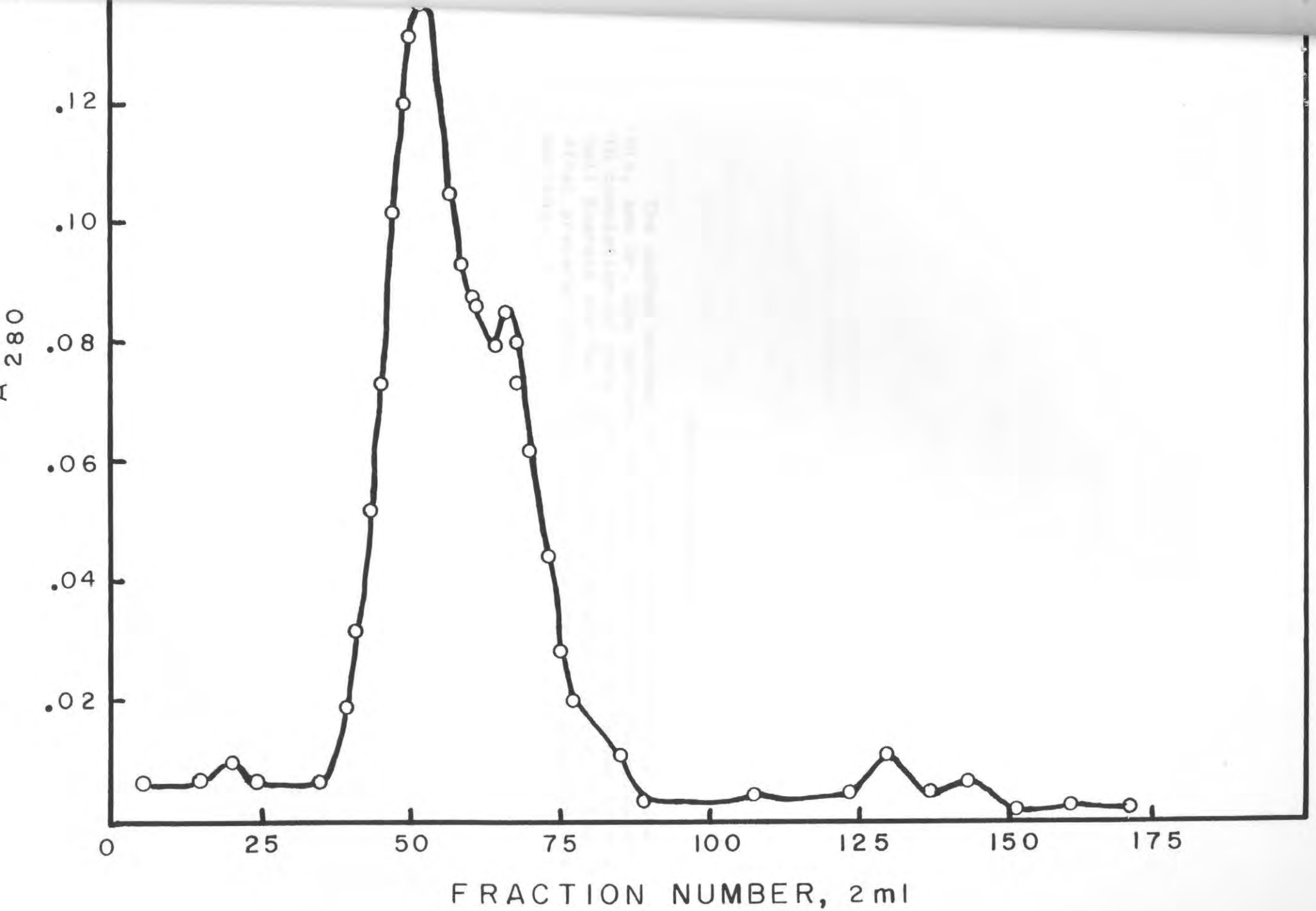
SP-Sephadex C-50 chromatography of charcoal-defatted, dimerized BPA. The resin bed was 12.5 x 1.5 cm in size and equilibrated with pouring buffer (0.09 M NaCl - 0.02 M Na acetate, pH 4.3). A 12.5 mg BPA sample was loaded onto the column with pouring buffer and collected in 2 ml fractions by

1. continuous gradient:

   125 ml of 0.09 M NaCl, pH 4.3

   125 ml of 0.43 M NaCl, pH 5.0

2. 75 ml of 0.43 M NaCl, pH 5.0
3. 75 ml of 0.64 M NaCl, pH 5.6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FRACTION NUMBER, 2 ml

Acknowledgments

The author wishes to thank Dr. Wayne Everett, Dr. Joe Nix, and Dr. Joe Jeffers for their instruction and guidance in completion of this study. Thanks is also extended to Mrs. Nell Everett for her patience and understanding during the final preparations of this paper into the early hours of the morning.

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