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Steric-Exclusion Chromatography at Pressures up to 3500 Kilograms per Square Centimeter

B. A. Bidlingmeyer[1] and L. B. Rogers

Department of Chemistry, Purdue University, Lafayette, Ind. 47907

THE PURPOSE of this study was to demonstrate the feasibility of doing steric-exclusion chromatography at pressures very much higher than are conventionally used. This exploratory investigation utilized bovine plasma albumin on a column of porous glass beads.

The native state of a protein is generally a compact, ordered form in water. The existence of this structure is believed to be due to the interaction of water with the hydrophobic side chains of the protein (*1*, *2*). Depending upon the magnitude of the pressure, protein denaturation may be retarded or accelerated (*3*, *4*). Pressure can also significantly influence the aggregation of macromolecules as has been demonstrated in ultracentrifugal studies (*5*, *6*).

Past investigations into pressure denaturations have generally relied upon classical methods to analyze the resulting solutions after pressure had been released (*7–9*). In addition to the usual parameters such as duration of the pressure and type of analysis, this approach introduced another experimental parameter, the time between pressure release and analysis.

One report by Aoki *et al.* (*9*) has described a boundary curve for the denaturation of bovine plasma albumin with respect to temperature and pressure. Their procedure was to expose the protein (1% solution) to a specified pressure and temperature in tris-boric acid buffer (pH = 8.9). After thirty minutes, the pressure was released and gel electrophoresis was performed to determine if any portion of the protein had denatured. The pressure–temperature points where any portion of the protein had denatured defined the boundary curve for denaturation. The authors pointed out that the boundary was not absolute, but depended upon the sample of bovine plasma albumin used. The denatured form was of a higher molecular weight, and the authors suggested that it was mostly a dimer.

The present communication describes the application of high-pressure steric-exclusion chromatography (*10*) in a brief investigation of the modification of bovine plasma albumin under pressure. A qualitative comparison with the results of Aoki and coworkers is presented.

EXPERIMENTAL

The Fraction V bovine plasma albumin was purchased from Armour Chemical Corp. (Chicago, Ill.) and was lot number D27309. The sample was defatted by the charcoal

[1] Present address, Standard Oil Company (Ind.), Standard Oil Research Center, P. O. Box 400, Naperville, Ill. 60540

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adsorption method (*11*), and the monomer peak was then isolated by gel filtration (*12*) in a 0.1*M* NaCl solution using Sephadex G-150 (Pharmacia Chemicals, Piscataway, N.J.). The monomer was collected, dialyzed against distilled water at 4 °C, and lyophilized. Disk gel electrophoresis on the final protein showed negligible presence of dimer.

For the chromatographic experiments, the protein was freshly dissolved (5% solution) in distilled, deionized water, and the solution was stored in ice until injected into the chromatograph. Elution was carried out using distilled deionized water that had been thermally degassed, using an infrared lamp, just before being pumped into the column.

The high-pressure chromatograph has been described elsewhere (*13*). Teflon (Du Pont) "O"-rings were used in the liquid sampling valve, in place of the usual rings of Buna-N, in order to minimize contamination of the protein. The pressure of the chromatograph was measured from a gauge calibrated in lb/in.^{2} and the values were converted to kg/cm^2 by using the equivalency 1 kg/cm^2 = 14.22 lb/in.^{2} and rounding to the nearest hundred. An R-4 refractometer (Waters Associates, Framingham, Mass.) was used to monitor the eluent.

The column packing was porous glass beads CPG-1250, 125–177 μ, mol wt range 40,000–550,000 (Corning Glass Works, Corning, N.Y.). The packing was degassed under vacuum for 1 hour and then packed as a slurry while vibrating the column. The pressure drop across the column itself was measured and found to be less than 70 kg/cm^2. After the high-pressure chromatographic experiments had been completed, the column packing had not diminished in height.

The protein samples were injected at atmospheric pressure and then the column was pressurized to the desired value. The necessary manipulations resulted in approximately 60 seconds of pressure application before flow started. Then, the valve at the exit end of the column was opened to allow elution to occur at about 0.50 ml/min. Elution itself generally required about 10 minutes. Hence, the protein was at the column pressure for a total of about 11 minutes. The eluent was collected in a buret and, every 0.50 ml, the input to the recorder was manually shorted to produce an event mark on the chart paper.

A molecular-weight calibration was not performed since the pressure effects on conventional standards were unknown. However, a blue dextran sample was eluted under 500 kg/cm^2 pressure to determine the void volume (4.0 ± 0.1 ml) and a sample of 3*M* sodium chloride was used to determine the interstitial volume (9.8 ± 0.1 ml).

RESULTS AND DISCUSSION

From the work of Aoki *et al.* (*9*), the pressure boundary should be 3000 kg/cm^2 at the temperature of the chromatograph, 23 °C. The high-pressure steric-exclusion chromatograms of bovine plasma albumin are shown in Figure 1. At 2800 kg/cm^2, there was a definite shift of the peak profile toward a higher molecular-weight species. At 3500 kg/cm^2, a maximum was clearly visible. These qualitative observations for the pressure-produced form agree rather closely with the Japanese report. The deviation from the exact value of Aoki *et al.* may have resulted from the fact that, as they point out, the boundary was not absolute. Furthermore, their pH was higher (8.9 *vs.* 7.0).

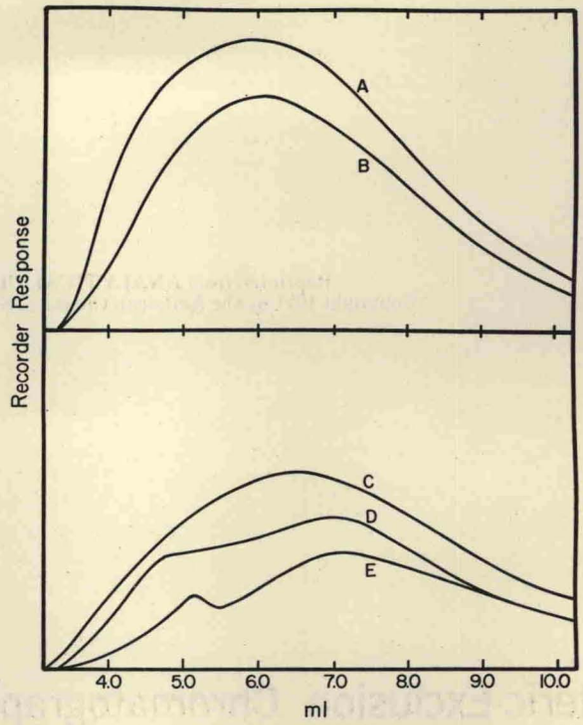

Figure 1. Effect of pressure upon elution profile of bovin plasma albumin

A. **700 kg/cm^2**
B. **1400 kg/cm^2**
C. **2100 kg/cm^2**
D. **2800 kg/cm^2**
E. **3500 kg/cm^2**

In our experiments, no attempt was made to study the effect of changing the time that the protein was maintained under pressure before the analysis was started. However, it seems reasonable to expect that longer times would have led to greater denaturation at a given pressure.

Further work is under way in high-pressure steric-exclusion chromatography to define its limitations. However, it is clear, even at this early stage, that high-pressure steric-exclusion chromatography using rigid porous glass beads will permit direct examinations of pressure effects on polymers of all types as well as on proteins. Comparison of pressure effects with those of other denaturants should be possible using this technique.

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