Chromatographic and Electrophoretic Fractionation of Soluble Proteins of Brain and Liver*

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The aim of the work the first stage of which is described here is to fractionate proteins of nervous tissue in order to compare these proteins with those of other organs to find specific qualitative differences, and to compare nervous tissue proteins of different species to find similarities. If unique proteins can be found which are common to nervous tissue of all species, then they may have some relation to function in the nervous system. This paper describes methods for fractionating proteins which are water-soluble. The general plan is to use the gentlest method of extraction first, followed by more drastic methods on the water-insoluble proteins.

In a previous paper (1) a method was described for fractionation of water-soluble proteins from liver by chromatography on diethylaminoethyl cellulose by elution with a parabolic gradient of water.

In this paper we show that starch gel electrophoresis of fractions of soluble proteins of brain, obtained after chromatography, is capable of giving a total of 70 to 100 resolvable bands, and that the resulting pattern, being highly reproducible, can be used to pick out particular proteins or groups of proteins which are characteristic of brain. It can also be used to follow the purification of specific proteins of brain when other methods of assay are not known.

EXPERIMENTAL PROCEDURE

Preparations of Soluble Proteins—All operations were done at 2-4°. Fresh whole beef brain was homogenized in a Waring Blender in 2 volumes of Buffer A (see description of gradient system below), and the homogenate was centrifuged for 1 hour at 100,000 × g. The supernatant fluid was equilibrated with Buffer A by dialysis or by passage through a column of Sephadex G-25 in equilibrium with the same buffer. Rat or rabbit brain extracts were prepared in the same manner. The distribution of protein in the homogenate of rat brain is shown in Table I.

About 10% of the wet weight of brain was protein and, of this total protein, about 20% was soluble under the conditions of preparation.

Operations of DEAE-cellulose Columns—The nine-chambered gradient device of Peterson and Sober (3) was used with combinations of three buffers in the chambers to give the required gradient shape. All buffers were prepared in glass-distilled water.

Buffer A consisted of Tris-phosphate, 5 mm in phosphate, pH 7.3. A 0.5 m stock solution, prepared with 34 ml of 14.7 m H3PO4 and 121 g of Tris per liter, was diluted to 5 m for use.

Buffer R was 1.0 m NaCl-50 m sodium phosphate, pH 7.3.

Buffer C, 1.0 m NaCl-50 m sodium phosphate, pH 6.2.

The buffers were mixed in the proportions shown in Table II in the nine chambers. Columns were eluted first with 100 to 150
ml (column 1 x 25 cm) of Buffer A, and the elution was then shifted to the gradient system. The high resistance to flow and variable flow rates characteristic of DEAE-cellulose were overcome by pumping the columns at constant flow rate with a peristaltic pump. The DEAE-cellulose was purified and prepared for use in the starting buffer, and the columns were packed as previously described (1). Most of the results in this paper are based on columns 1 x 25 cm packed with 4 to 5 g of DEAE-cellulose.

The buffer system was designed to maintain the pH at 7.2 to 7.3 until about half the gradient volume had passed through the column, and then to lower it gradually to 6.2, since separations of several enzymes were better when the pH was greater than 7 during the early part of the elution. Generally 10 mM 2-mercaptoethanol was added to all the buffers in order to stabilize the enzyme activities. Columns (1 x 25 cm) were eluted at a flow rate of about 20 ml per hour. As a general rule, less than 100 mg of soluble proteins per g of DEAE-cellulose, dry weight, were fractionated on a column. Fig. 1 shows the shape of the salt gradient used and the agreement between actual and calculated salt concentrations in the fractions.

### Table I

**Preparation and chromatography of rat brain soluble proteins**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume ml</th>
<th>Weight g</th>
<th>Per cent of wet weight</th>
<th>Protein mmoles/hr</th>
<th>% Specific activity</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>244</td>
<td>7.7</td>
<td>9.2</td>
<td>2,040</td>
<td>200</td>
<td>100</td>
</tr>
<tr>
<td>Insoluble</td>
<td>5.2</td>
<td>1.6</td>
<td>6.2</td>
<td>1,520</td>
<td>932</td>
<td>75</td>
</tr>
<tr>
<td>Soluble</td>
<td>162</td>
<td>1.5</td>
<td>1.8</td>
<td>1,590</td>
<td>1,026</td>
<td>75</td>
</tr>
<tr>
<td>Recovered</td>
<td>142</td>
<td>1.31</td>
<td>77.0</td>
<td>1,340</td>
<td>1,026</td>
<td>75</td>
</tr>
<tr>
<td>from column</td>
<td>0.89t</td>
<td></td>
<td></td>
<td>930</td>
<td>19,000</td>
<td>70</td>
</tr>
</tbody>
</table>

* Starting with 94.1 g of brain, wet weight.
† Not an accurate value because of difficulty in pipetting.
‡ Recovery from the column was 68%.

### Table II

**Gradient system for chromatography**

The compositions of Buffers A, B, and C are described under "Experimental Procedure."

<table>
<thead>
<tr>
<th>Chamber</th>
<th>Amount in chamber of</th>
<th>Chloride concentration mM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Buffer A ml</td>
<td>Buffer B ml</td>
</tr>
<tr>
<td>1</td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>77</td>
<td>3.0</td>
</tr>
<tr>
<td>3</td>
<td>74</td>
<td>6.0</td>
</tr>
<tr>
<td>4</td>
<td>74</td>
<td>9.0</td>
</tr>
<tr>
<td>5</td>
<td>76</td>
<td>3.5</td>
</tr>
<tr>
<td>6</td>
<td>64</td>
<td>15.0</td>
</tr>
<tr>
<td>7</td>
<td>61</td>
<td>18.0</td>
</tr>
<tr>
<td>8</td>
<td>58</td>
<td>21.0</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>77.0</td>
</tr>
</tbody>
</table>

Assays on Fractions—Protein was assayed by the method of Lowry et al. (4) after the protein was precipitated with trichloroacetic acid and redissolved in 1 N NaOH in order to avoid interference by mercaptoethanol. In some cases protein was also assayed by absorbance at 280 mμ and by native fluorescence when more sensitivity was needed (activation at 280 mμ and fluorescence at 350 mμ). Enzyme activities in the fractions were assayed by the methods described previously (1). Several others were assayed under the conditions shown in Table III.

Pooling and Concentration of Fractions—The contents of tubes from the chromatography were pooled to give a total of 15 fractions, which corresponded to the resolving power of the column (see "Results"). Each pooled fraction was concentrated 20 to 100 times by pressure dialysis to give a protein concentration of about 20 mg per ml in a buffer of the following composition: 0.1 M NaCl, 0.05 M Tris-chloride, 0.01 M potassium phosphate, and 0.001 M sodium Versenate, pH 7.5.

Electrophoresis—Vertical starch gel electrophoresis was performed by the method of Smithies (2) in a commercially available apparatus (Otto Hiller, Madison, Wisconsin) with the starch prepared by Connaught Laboratories. Of the buffer systems tried, the one which was found to give the greatest number and sharpest bands was the discontinuous system of Poulik (5); 5 mM Tris-citrate, pH 8.6, was used as the gel buffer, and 0.3 M sodium borate, pH 8.3, as the electrode buffer. The resolution, as estimated by number and sharpness of bands, was greater at room temperature than in the cold. Since the purpose of the electrophoresis of the column fractions was to compare patterns and to follow purification of specific proteins, it was thought that it should be carried out under conditions resulting in maximum resolving power. Therefore the gels were run at room temperature 4 to 5 hours at 250 volts. The resolution was also improved

1 With fractions of beef brain, the Lowry method gave consistently higher recoveries of protein (70 to 90%) than did absorbance at 280 mμ (60 to 80%) or fluorescence (60 to 70%).

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**Fig. 1. Agreement between calculated (solid line) and measured (filled circles) chloride concentration in fractions from a DEAE-cellulose column (1 x 25 cm) loaded with brain proteins and eluted with the nine-chambered gradient system described under "Experimental Procedure."**
by adding 10 mM 2-mercaptoethanol to the gel buffers. The gels were sliced and the bottom halves were stained in Buffalo black (0.5% dye in 5% acetic acid) for 30 minutes, and then destained in an electrophoretic destainer.

RESULTS

Results of Chromatography—Fig. 2 shows chromatograms of rabbit liver and brain soluble proteins with patterns of several enzymes in the fractions. Protein and enzyme activity recoveries and patterns were similar when 1 mg or 100 mg of beef brain soluble proteins were run, with the described gradient system, on columns 1 X 25 cm. A similar pattern was obtained when 1 g of a soluble protein preparation was run on a column 2.5 X 35 cm with double the gradient volume. Variation of flow rate between 13 and 52 ml per hour for columns 1 X 25 cm did not affect the protein patterns or positions of enzyme activity peaks (6-phosphogluconate dehydrogenase and acid phosphatase). Repetition of chromatography on samples of the same beef brain preparation or on separate rat brain preparations gave highly reproducible results, as judged by protein patterns and positions of elution of enzyme activities. Elution volumes of peaks of enzyme activities did not vary more than ±15 ml out of a total gradient volume of 720 ml.

Recoveries of protein and enzyme activities for liver and brain are shown in Table IV. Protein recoveries were consistently lower for brain than for liver, probably reflecting the greater proportion of lipoproteins in brain. The resolving power of the chromatography was measured by the spread of single enzyme peaks as shown in Table V. For columns 1 X 25 cm, the spread amounted to 1/15 to 1/25 of the total gradient volume.

Comparison of Brain and Liver Patterns—Comparison of patterns of protein in chromatograms of liver and brain preparations of rabbit (Fig. 2) showed that brain contained more protein which was bound most tightly to the DEAE-cellulose (Fractions 100 to 120 eluted by 0.2 to 0.4 M chloride) than did liver. This...
difference held true for three other species: rat, monkey, and beef (Table VI). Brain protein patterns were similar for several species (Fig. 3). Multiple form enzymes such as acid phosphatase and glucose 6-phosphate dehydrogenase showed similar patterns in liver and brain of the same species but different proportions of the forms, and other single peak enzymes such as 6-phosphogluconate dehydrogenase and α-glycerol phosphate dehydrogenase were eluted at identical positions in brain and liver chromatograms (Fig. 2). On the other hand, for brains of different species, specific enzymes were eluted at different positions (Figs. 4 and 5), some enzymes (6-phosphogluconate dehydrogenase) showing less variation in elution position in different species than others (acid phosphatase).

**TABLE VI**

Percentage of protein eluted

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Before gradient</th>
<th>0.2 to 0.4 M chloride</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat Brain</td>
<td>21.8 %</td>
<td>25.7 %</td>
</tr>
<tr>
<td>Rat Liver</td>
<td>33.4 %</td>
<td>15.6 %</td>
</tr>
<tr>
<td>Rabbit 1 Brain</td>
<td>28.4 %</td>
<td>35.1 %</td>
</tr>
<tr>
<td>Rabbit 1 Liver</td>
<td>58.0 %</td>
<td>10.9 %</td>
</tr>
<tr>
<td>Rabbit 2 Brain</td>
<td>24.7 %</td>
<td>30.3 %</td>
</tr>
<tr>
<td>Rabbit 2 Liver</td>
<td>48.0 %</td>
<td>11.7 %</td>
</tr>
<tr>
<td>Beef brain</td>
<td>31.5 %</td>
<td>29.3 %</td>
</tr>
<tr>
<td>Monkey brain</td>
<td>18.5 %</td>
<td>33.8 %</td>
</tr>
</tbody>
</table>

**Starch Gel Electrophoresis of Fractions**—It was shown earlier (1, 6) that after liver or brain proteins were fractionated on DEAE-cellulose, separately rechromatographed fractions appeared at the expected positions. In order to judge the effect of electrophoresis on brain proteins, the following rechromatography experiment was done. The eluate from the chromatography of beef brain soluble proteins was divided into five fractions, which were concentrated and subjected to starch gel electrophoresis. After electrophoresis the proteins from the entire gel, for each chromatographic fraction, were eluted and...
rechromatographed as described under "Experimental Procedure." The result (Fig. 6) showed that electrophoresis did not alter the chromatographic behavior of the soluble proteins.

Contents of tubes from chromatography of soluble proteins of beef brain were pooled into 15 fractions as shown in Fig. 7, corresponding to the resolving power of the chromatography as estimated by peak widths of enzyme activities. The division into fractions was arbitrary and was not intended to correspond to either protein or enzyme peaks, since in general enzyme peaks did not correlate with protein peaks, and furthermore each of the fractions was a complex mixture. The fractions were concentrated as described in "Experimental Procedure" and subjected to starch gel electrophoresis.

The electrophoretic pattern of the 15 beef brain fractions is shown in Fig. 8. At least 200 separate bands could be counted in the pattern. The patterns for a given type of soluble protein preparation were highly reproducible with regard to relative positions of the bands. Figs. 9 and 10 compare patterns of soluble proteins from rat brain and liver. There were several intensely stained, fast moving bands in Fractions 13 to 15 in brain which were weaker or missing in liver (e.g., those at 0.9 in Fractions 13 and 14, and those at 1.1 and 1.4 in Fraction 15 of brain). Most striking was the apparently complete absence of the band at 1.4 (Fraction 15) in liver. A band at this relative position was seen in the beef brain pattern (Fig. 8), and it was also seen in patterns of brain of all other species examined (rabbit, monkey, human), but was absent in liver of these species. On the other hand, there were many similar bands in brain and liver which could be seen in Figs. 9 and 10; for example, those in both brain and liver at 0.2 in Fraction 1, 0.7 in Fraction 2, 0.9 in Fraction 4, 0.45 in Fraction 6, 0.5 in Fractions 9 and 10, and 0.32 in Fraction 14.

**Discussion**

On the basis of protein and enzyme recoveries from the columns and the localization of specific enzyme activities in the fractions, the described method was valid for fractionation of brain and liver soluble proteins with minimal loss. A single protein localized in the fractions (for example, as measured by 6-phosphogluconate dehydrogenase or α-glycerol phosphate dehydrogenase activity) was eluted in 1/15 to 1/25 of the total gradient volume for columns 1 X 25 cm, and in 1/12 of the volume for columns 2.5 X 35 cm, indicating the resolution of the chromatography.

The method was reproducible as shown by similar chromatograms from the same beef brain preparation or from separate rat brain preparations. The reproducibility depended mainly on two factors which must be controlled, i.e. type of adsorbent and the shape of the gradient, and depended little on rate of elution, amount of protein, and size of column.

The method was shown to be adaptable to direct comparisons of patterns of protein mixtures, for example from brain and liver, especially if the columns were run simultaneously from the same gradient. High purifications of specific proteins are possible because of the resolution attained with the improved gradient system for eluting the columns. For example, 6-phosphogluconate dehydrogenase was purified 20- to 30-fold during the chromatography (Table I). The method could be scaled up for preparative work since it was shown that 1 g of brain protein could be chromatographed on a column 2.5 x 35 cm with small loss in resolution.

Comparison of chromatographic patterns of brain and liver revealed marked differences. Preparations of brain soluble proteins contained more protein binding most tightly to DEAE-cellulose than those from liver. Furthermore, in brain there was less material that did not bind at all to the DEAE-cellulose (Fig. 3 and Table VI). These differences were seen consistently between brain and liver preparations from several species, i.e. rabbit, monkey, and beef (Fig. 3). For brain and liver of the same species, specific enzymes seemed to coincide in elution position, although for some multiple form enzymes (e.g. glucose...
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FIG. 8 (upper). Starch gel electrophoretic pattern of 15 concentrated fractions from DEAE-cellulose chromatography of beef brain soluble proteins. Fractions 1 to 15 are shown from left to right. The last sample (Fraction 16) is crystalline bovine plasma albumin.

FIG. 9 (lower left). Rat liver two-dimensional pattern as described in the text. The horizontal scale is chromatographic fraction number, and the vertical scale is distance of migration relative to the salt front in the discontinuous buffer system.

FIG. 10 (lower right). Rat brain two-dimensional pattern as described in the text. The horizontal scale is chromatographic fraction number, and the vertical scale is distance of migration relative to the salt front in the discontinuous buffer system.

6-phosphate dehydrogenase and acid phosphatase) relative peak heights varied for the different forms.

Direct starch gel (7) or polyacrylamide gel (8) electrophoresis of proteins extracted from brain has been shown to give 15 to 30 bands. The two-dimensional method described here gave a total of about 200 bands on the stained gels for whole brain soluble proteins. If it is assumed that a band corresponding to a given protein was repeated in 2 or 3 adjacent fractions of the chromatographic column, then the actual number of different resolvable proteins was 70 to 100. The improved resolution may be due to any of several factors. Overlapping of bands in one-dimensional electrophoresis may obscure some bands and lead to a diffuse pattern, or there may be interactions among proteins which are minimised when fractions are separated by chromatography before electrophoresis. The main advantage of the two-dimensional method is that it permits a direct com-
comparison of highly resolved patterns of complex mixtures of proteins. It can be used to select particular proteins of brain for further study on the basis of differences; for example, between patterns of the proteins of brain and other organs such as liver, or among areas of brain, or among stages of development of brain.

It was evident, when the two-dimensional patterns of soluble proteins from brain and liver were compared, that brain contained at least two proteins which were in lower quantity or absent in liver. These were relatively acidic and probably small in size on the basis of their chromatographic and electrophoretic behavior. The faster moving band of these two was present in brain of all species examined and was apparently absent from their livers. It can be estimated that for this band to have been visibly absent in the liver pattern, the concentration of this protein in liver must have been less than 1% of that in brain. It was not surprising to find similar proteins characteristic of brain of different species. Such proteins, for example the acidic ones shown here, may be related to the unique function and organization of nervous tissue, and therefore it would be of interest to purify them for further study. The techniques described here can serve not only to pick out such proteins, but also to purify and assay them.

SUMMARY

A method for producing two-dimensional protein patterns from water-soluble protein extracts of brain and liver gave 70 to 100 resolvable protein bands. The method consisted of chromatography on diethylaminoethyl cellulose with an improved gradient system, followed by starch gel electrophoresis of the fractions. The chromatographic step showed that brain contained, in general, a greater quantity of acidic proteins than liver. The two-dimensional patterns showed the presence in brain of proteins which were absent or in small amount in liver. Similar proteins were found in brains of all species examined.

REFERENCES

Chromatographic and Electrophoretic Fractionation of Soluble Proteins of Brain and Liver
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