The Activity of Human Placenta Microsomes and Brain Particles in Blood Coagulation*

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SUMMARY

1. Human placenta microsomes active in blood coagulation have been prepared by a combination of differential centrifugation, density gradient centrifugation, and extraction with butanol-benzene mixtures. Human brain particles have been prepared from acetone-dried brain by extraction with sodium chloride solutions, differential centrifugation, and extraction with butanol-benzene mixtures.

2. Placenta microsomes and brain particles appear to function as enzyme in the development of coagulant activity from the bovine serum fraction.

3. The coagulant activity, which develops in reaction mixtures containing placenta microsomes, calcium, and bovine serum eluate, rapidly disappeared on storage at 4°C. In similar reaction mixtures containing purified brain particles, less than one-half of the coagulant activity could be recovered in the sediment after washing with buffer containing calcium, but from 75 to 100% could be recovered in the supernatant fluid after washing with ethylenediaminetetraacetate.

4. Purified brain particles bind protein from the bovine serum eluate nonspecifically and bind activated Factor X, but do not bind nonactivated Factor X.

In previous studies, it was shown that bovine lung microsomes appear to function as enzyme in blood coagulation (1). In past and present work the term "microsomes" is used in the operational sense only and does not imply functional or morphological characteristics. Human placenta and brain are other well recognized sources of "tissue thromboplastin" and in the present study these two organs were examined by using the approach previously employed with bovine lung. Preparations active in blood coagulation, obtained from extracts of fresh placenta and acetone-dried brain, were found to function in essentially the same manner as bovine lung microsomes in blood coagulation.

EXPERIMENTAL PROCEDURE

Assay of coagulant activity, density gradient centrifugation, and chemical analyses were performed as previously described (1).

Preparation of Placenta Microsomes—Human placenta was treated in a manner similar to that reported for bovine lung, except that the placenta was disintegrated in the Waring Blender in a volume of 0.15 M NaCl equal to 1.5 times its weight, and then centrifuged in an angle rotor at 5,000 × g for 30 min to remove the cell debris. The supernatant fluid ("original") was then centrifuged at 78,480 × g for 45 min to collect the "crude microsome" fraction. The remainder of the procedure, including purification of the microsomes in a Urokon density gradient, was identical with that reported for bovine lung (1).

Preparation of Human Brain Particles—The starting material for preparation of human brain particles was a 0.15 M NaCl extract of a powder of acetone-dried human brain. The gray matter of brain was kneaded with acetone at room temperature until the tissue became granular. The mixture was then stirred in a Waring Blender, and the acetone removed by filtration. The extraction with acetone in the Blender was repeated six times. The final powder was air-dried and stored at −15°C. The yield from one human brain was 143 g. Purified brain particles were prepared as outlined in Fig. 1.

RESULTS

Properties of Purified Placenta Microsomes—Data on purification of microsomes from a placenta weighing 300 g are presented in Table I. The over-all purification was 38-fold from the original but was only about 6-fold from the crude microsomes step. This was also observed with bovine lung (1). The purified microsomes contained 7.9% nitrogen, 1.8% phosphorus, and 6.7% cholesterol. Of the total phosphorus, 94% was phospholipid phosphorus. The composition of the placenta microsomes was calculated to be 49% protein, 42% phospholipid phosphorus, and 7% cholesterol. Extracted phospholipids were examined by

1 Urokon sodium is sodium 3-acetamido-2,4,6-triiodobenzoate prepared by Mallinckrodt. I am indebted to Mr. Robert Robertson for generous supplies of this material.

The preparation of acetone-dried brain is a previously unpublished method of Dr. Harold A. Wurzel of the University of Pennsylvania.
chromatography on silicic acid-impregnated paper, and mixed phospholipids were present, as previously found with the bovine lung microsomes (1).

Density gradient centrifugation of the purified microsomes showed that the material was polydisperse when studied by zone centrifugation. On isopycnic density gradient centrifugation, a single band was present, containing material from density about 1.10 g per ml to 1.17 g per ml.

Properties of Purified Brain Particles—Table II shows data from a typical preparation of purified brain particles. Overall purification based on nitrogen analysis was about 43-fold, while that based on phosphorus analysis was 10-fold. The yield of coagulant activity after centrifugation of the suspension of crude particles at 20,000 × g was only about 60% of that present prior to this step. Requirement for a combination of soluble and insoluble components for full activity could not be shown since mixtures of the soluble and concentrated particles fraction did not show coagulant activity greater than the sum of the individual activities of these components.

The final product contained 7.0% nitrogen and 2.2% phosphorus. No cholesterol was present. All of the phosphorus was present as phospholipid phosphorus. The composition of the particles was calculated to be 44% protein and 56% phospholipid. On zone centrifugation in sucrose density gradients, the brain microsomes were polydisperse and sedimented more rapidly than did those from lung or placenta. On isopycnic density gradient centrifugation, the final product sedimented as a rather narrow band of density from about 1.08 to 1.10 g per ml. The extracted phospholipids were chromatographed on silicic acid-impregnated paper, and a mixture of phospholipids was found, as with lung or placenta microsomes.

### Table I

<table>
<thead>
<tr>
<th>Purified brain (large particles)</th>
<th>Crude microsomes supernatant</th>
<th>Crude microsomes</th>
<th>Centrifugation 1</th>
<th>Microsomes 1</th>
<th>Centrifugation 2</th>
<th>Microsomes 2</th>
<th>Centrifugation 3</th>
<th>Microsomes 3</th>
<th>Butanol-benzene extraction</th>
<th>Butanol-benzene extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume</td>
<td>550</td>
<td>500</td>
<td>25</td>
<td>150</td>
<td>25</td>
<td>150</td>
<td>25</td>
<td>150</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Total phosphorus</td>
<td>48,300</td>
<td>18,400</td>
<td>6,500</td>
<td>2,200</td>
<td>2,700</td>
<td>18,000</td>
<td>1,400</td>
<td>500</td>
<td>2,000</td>
<td>4,000</td>
</tr>
<tr>
<td>Yield</td>
<td>100</td>
<td>0</td>
<td>6,800</td>
<td>400</td>
<td>2,100</td>
<td>28,500</td>
<td>2,000</td>
<td>0</td>
<td>1,970</td>
<td>4,000</td>
</tr>
<tr>
<td>Total units</td>
<td>9,900</td>
<td>0</td>
<td>42,000</td>
<td>400</td>
<td>32,200</td>
<td>28,500</td>
<td>2,000</td>
<td>320</td>
<td>2,200</td>
<td>4,000</td>
</tr>
<tr>
<td>Specific activity units/μg phosphorus</td>
<td>0.2</td>
<td>0</td>
<td>1.3</td>
<td>1.4</td>
<td>1.6</td>
<td>1.6</td>
<td>0.2</td>
<td>7.6</td>
<td>1.9</td>
<td>3.3</td>
</tr>
</tbody>
</table>

* Yield calculated separately for differential centrifugation and for density gradient centrifugation and butanol-benzene extraction.

**Fig. 1. Flow sheet for preparation of purified brain particles.**
Phosphorus content of microsomes.

The maximum concentration of placenta microsomes was 0.67 pg of phosphorus per ml.

The optimal concentration of calcium was about pH 8.0 with brain particles, and at pH 8.5 to 9.0 with placenta microsomes.

Maximal activity occurred at about pH 8.0 with brain particles, and at pH 8.5 to 9.0 with placenta microsomes.

The maximum concentration of placenta microsomes was 0.95 pg of phosphorus per ml, and that of brain microsomes was 0.67 pg of phosphorus per ml.

The reaction mixture contained 0.1 ml of brain particles or placenta microsomes on the rate of formation of coagulant activity. The reaction mixture contained 0.1 ml of brain particles or placenta microsomes on the rate of formation of coagulant activity. The reaction mixture contained 0.1 ml of brain particles or placenta microsomes on the rate of formation of coagulant activity. The reaction mixture contained 0.1 ml of brain particles or placenta microsomes on the rate of formation of coagulant activity.

The curves have the shape of a rectangular hyperbola, as expected if bovine serum fraction function as enzyme. Further experiments, it was found that the yield of coagulant activity was independent of the amount of lung microsomes or brain particles added to the reaction mixture over a 5-fold range, suggesting the tissue extracts were acting as enzyme.

Further support for the role of the tissue preparations as enzyme and of bovine serum fraction as substrate was obtained from kinetic studies. The effect of concentration of placenta microsomes or brain particles on the initial rate of formation of coagulant activity is shown in Fig. 2. Here the initial rate is directly proportional to the concentration of the tissue preparations, indicating they are functioning as enzyme. The effect of bovine serum fraction concentration on the initial rate of formation of coagulant activity was also determined. Data from such experiments with placenta and brain preparations are presented in Table II. The curves have the shape of a rectangular hyperbola, as were expected if bovine serum fraction were functioning as substrate.

The effect of pH on the initial rate of formation of coagulant activity is presented in Fig. 4. Maximal activity occurred at about pH 8.0 with brain particles, and at pH 8.5 to 9.0 with placenta microsomes. The optimal concentration of calcium was about 0.006 M for the reactions involving either brain particles or placenta microsomes.

Role of Phospholipid—Previous studies with lung microsomes showed that the intrinsic phospholipid of the microsomes could function as phospholipid in the assay system employed in these studies. Similar studies were carried out with placenta microsomes heated at 60° for 60 min, which reduced the activity of these microsomes in the one-stage assay to about 2% of the original amount. It was necessary to heat brain particles at 70° for 2 hours to reduce the activity in the one-stage assay to 2% of the original. Isopycnic density gradient centrifugation of the

**Fig. 2.** The effect of concentration of purified brain particles and placenta microsomes on the rate of formation of coagulant activity. The reaction mixture contained 0.1 ml of brain particles or placenta microsomes at the concentrations noted on the abscissa, 0.1 ml of bovine serum fraction containing 100 units of potential activity in 0.78 mg of protein, and 0.1 ml of 0.025 M CaCl₂. The vessels were incubated at 37° for 1 min after adding the calcium, and then were assayed without dilution in the simplified system previously described (1). , brain particles; X, placenta microsomes. The maximum concentration of placenta microsomes was 0.95 µg of phosphorus per ml, and that of brain microsomes was 0.67 µg of phosphorus per ml.
heated placenta microsomes and brain particles was carried out and the pattern was unchanged from that of unheated preparations. There was no free phospholipid apparent either in the heated or unheated preparations. The ability of heated placenta microsomes and brain particles to function as phospholipid in blood coagulation is illustrated in Fig. 5. The placenta microsomes are not as effective as the brain particles in providing phospholipid in the assay system used to detect Factor X.

**Nature of Coagulant Activity**—The coagulant activity formed in reaction mixtures containing placenta microsomes or brain particles appeared to be identical with that formed when Russell's viper venom was used. Bovine serum fraction fully activated with either tissue preparation developed no further activity on incubation with Russell's viper venom. Conversely, bovine serum fraction fully activated with Russell's viper venom could not be further activated with either tissue preparation.

**Physical Changes in Placenta Microsomes and Brain Particles on Incubation with Serum Fraction**—In the previous study on lung microsomes, evidence was obtained for complex formation between lung microsomes, calcium, and protein from the bovine serum fraction. These studies were extended to placenta microsomes and brain particles. In these experiments, a mixture of placenta or brain preparation, bovine serum fraction, and calcium were incubated at 37° for a sufficient time to permit full development of the coagulant activity of the bovine serum fraction. The reaction mixture was then chilled in melting ice, and centrifuged in the cold to collect the particles. These were then washed once or twice and resuspended in a small volume of buffer solution. The various fractions were tested for coagulant activity, and the final suspension was subjected to density gradient centrifugation or to chemical analysis. Unlike lung microsomes, placenta and brain preparations gave poor recoveries of coagulant activity. With placenta microsomes very little coagulant activity could be detected in an aliquot of the reaction mixture maintained in melting ice for the period required for the experiment, although full activity was present in the reaction mixtures tested immediately after the incubation period. In a typical experiment with placenta microsomes only 20% of the original activity was present in the sediment and no activity was present in the supernatant fluid after centrifugation of the reaction mixture for 30 min. After one wash of the sediment with buffer containing calcium, only 5% of the original activity remained. Washing the sediment with EDTA did not elute any significant activity. On density gradient centrifugation of the final sediment washed with buffer containing calcium, a single, narrow band was present, with density approximately 1.18. The microsomes prior to incubation showed a broader band with an average density of 1.14 g per ml. Because of the marked instability of the coagulant activity in this system, no further studies were carried out.

Brain particles were studied in a similar manner. Data on the coagulant activity of brain particles incubated in a complete reaction mixture and appropriate controls are presented in Table III. Incubation of brain particles with bovine serum fraction and calcium led to the development of full coagulant activity which was completely removed from the supernatant fluid on centrifugation. The sediment, after one wash with buffer solution containing calcium, had about 23% of the coagulant activity originally present. Incubation of the brain particles with bovine serum fraction alone or calcium alone did not lead to the development of significant coagulant activity. The nitrogen to phosphorus molar ratio of the sediment from the complete reaction mixture was increased from 6.4 to 12.3; that of the reaction mix-

![Fig. 3. The effect of bovine serum fraction concentration on the rate of formation of coagulant activity. The reaction mixture contained 0.1 ml of bovine serum fraction at the concentrations noted on the abscissa, 0.1 ml of brain particles or placenta microsomes, and 0.1 ml of 0.025 M CaCl₂. The maximum concentration of bovine serum fraction was 100 units of potential activity in 0.78 mg of protein in both experiments. The brain particle suspension contained 0.44 μg of phosphorus per ml. The placenta microsomes contained 0.53 μg of phosphorus per ml. ○, brain particles; X, placenta microsomes.](http://www.jbc.org/)
particles, the reaction did not go to completion in 5 min, since phosphorus. After a 5-min incubation period at 37°, the coagulant activity in the three vessels was 100, 80, and 35% of maximum. Apparently with the lower concentrations of brain were prepared which were similar to those just described except coagulant activity bound was studied. Three reaction mixtures protein to the particles, as well as the marked degree of binding which occurs in the complete system.

The density of the particles incubated without calcium was increased to an average of 1.11 g per ml, as were those incubated without bovine serum fraction. The particles from the complete reaction mixture formed a dense narrow band, while those from the other reaction mixtures gave a broader band. These results indicate some binding of calcium and nonspecific binding of the other reaction mixtures giving a broader band. These results indicate that protein is bound to the brain particles in the system yielding coagulant activity, but there is also nonspecific binding which occurs in the absence of calcium.

In Fig. 6 are shown the results of density gradient centrifugation of the sediment from a duplicate experiment of the kind just described. The density of the sediment from the complete reaction mixture was increased from about 1.09 to 1.14 g per ml. The density of the particles incubated without calcium was increased to an average of 1.11 g per ml, as were those incubated without bovine serum fraction. The particles from the complete reaction mixture formed a dense narrow band, while those from the other reaction mixtures gave a broader band. These results indicate some binding of calcium and nonspecific binding of protein to the particles, as well as the marked degree of binding which occurs in the complete system.

The effect of concentration of brain particles on the amount of coagulant activity bound was studied. Three reaction mixtures were prepared which were similar to those just described except that the quantity of brain particles was 50, 30, and 10 μg of phosphorus. After a 5-min incubation period at 37°, the coagulant activity in the three vessels was 100, 80, and 35% of maximum. Apparently with the lower concentrations of brain particles, the reaction did not go to completion in 5 min, since incubation of similar vessels for a longer time increased the yield. In all three vessels, all of the coagulant activity which developed was bound to the particles, with none appearing in the supernatant fluid.

In other experiments evidence was obtained that the protein bound to the brain particles in the experiments where calcium was omitted was not coagulant protein because after removal of the particles it was possible to develop full coagulant activity in the supernatant fluid with either Russell's viper venom or brain particles. Further, no increase occurred in the coagulant activity of the particles recovered in these experiments when they were subsequently incubated with calcium.

In the experiment presented in Table IV, the effect of washing brain particles, after incubation with bovine serum fraction and calcium, with calcium and with EDTA solutions was studied. As with lung microsomes, the coagulant activity bound to the brain particles could be eluted with EDTA solutions. It is of interest that in all five of the experiments in which EDTA washes were employed, more activity was recovered in the EDTA wash than could be detected in the sediment washed with calcium solutions. The activity present in the suspension of the sediment washed with calcium solutions was 20 to 45% of that found in the EDTA washes, suggesting that all of the bound activity is not available for reaction in the coagulation system.

Isopycnic density gradient centrifugation demonstrated again the marked increase in density of the brain particles after incubation with bovine serum fraction and calcium, and the slight increase in density of incubation of the brain particles with bovine serum fraction alone. The particles washed with EDTA sedimented as a broad band with an average density of about 1.12, which is less than that of the particles from the complete reaction mixture (density 1.14 g per ml) but still greater than that of the original material.
Fig. 6. Density gradient centrifuge patterns of brain particles treated as described in Table III. These are negative images of plastic centrifuge tubes (1.2 x 5 cm) illuminated from the side. Tubes 1, 2, and 3 contained, respectively, sediments from vessels identical with Vessels 1, 2, and 3 of Table III. Tube 4 contained untreated brain particles. The density markers are 1.09, 1.13, and 1.15 g per ml.

### Table III

**Binding of coagulant activity to brain particles**

The reaction mixture for Vessel 1 consisted of 3.5 ml of bovine serum fraction containing 3,500 units of potential activity in 28 mg of protein, 1.0 ml of brain particles containing 44 μg of phosphorus suspended in 0.15 M NaCl, and 0.8 ml of 0.1 M CaCl₂ in 0.01 M Tris-chloride buffer at pH 7.4. In Vessel 2, 0.01 M Tris-chloride buffer at pH 7.4 was substituted for the CaCl₂, and in Vessel 3, 3.5 ml of Tris-NaCl buffer at pH 7.4 was substituted for the bovine serum fraction. The vessels were incubated at 37° for 5 min, and then chilled in melting ice. A 0.5-ml sample was removed from each vessel, and the remainder was centrifuged at 40,000 rpm in the SW 39L rotor of the Spinco model L centrifuge. The supernatant fluid was decanted and the sediments were resuspended in 4.8 ml of a solution of 0.15 M NaCl and 0.015 M CaCl₂ at pH 7.4. The final sediments were examined by isopycnic density gradient centrifugation in sucrose density gradients as shown in Fig. 6.

### Table IV

**Effect of EDTA on binding of coagulant activity to brain particles**

The reaction mixture in Vessels 1 and 2 consisted of 3.6 ml of bovine serum fraction containing 3,600 units of potential activity in 28 mg of protein, 0.4 ml of brain particles containing 32 μg of phosphorus suspended in 0.15 M NaCl, 0.5 ml of 0.1 M Tris-chloride at pH 7.4, and 0.8 ml of 0.1 M CaCl₂. In Vessel 3 the CaCl₂ solution was replaced by 0.8 ml of 0.01 M Tris-chloride buffer at pH 7.4. The vessels were incubated at 37° for 5 min and then chilled in melting ice. A 0.5-ml sample was withdrawn from each vessel, and the remainder was centrifuged at 40,000 rpm in the SW 39 L rotor of the Spinco model L centrifuge for 30 min. The supernatant fluids were decanted. The sediments from Vessels 1 and 3 were suspended in 4.8 ml of 0.15 M NaCl and 0.015 M CaCl₂ at pH 7.4. The sediment from Vessel 2 was suspended in 4.8 ml of 0.01 M EDTA in 0.15 M NaCl. The vessels were again centrifuged as before. The supernatant fluids (wash) were decanted and the sediments were suspended in 0.6 ml of the NaCl-CaCl₂-Tris solution previously used. Coagulation assays were performed with the simplified system.

### DISCUSSION

In the present study, the purification procedures were designed to yield highly active material which was suitable for study by density gradient centrifugation, as was achieved previously with bovine lung. Satisfactory preparations were obtained from both human placenta and brain. The human placenta micro-
somes and human brain particles both appear to function in blood coagulation in a manner quite similar to the bovine lung microsomes. All three preparations are composed principally of protein and mixed phospholipid. Cholesterol was present in the microsomes but was absent from the brain particles which were derived from acetone-dried brain. The brain particles thus contained relatively more phospholipid than the other preparations and had a lower density. Hecht, Cho, and Seegers (2) prepared brain extracts active in blood coagulation and found they were composed of protein-free lipids. Their preparation was obviously quite different from that studied here.

In contrast to the previous results with lung microsomes, the recovery of coagulant activity after centrifugation of reaction mixtures containing placenta microsomes or brain particles was low, and all of the coagulant activity was bound to the particles, with none in the supernatant fluid. In the experiments with placenta microsomes the coagulant activity could not be recovered by washing with EDTA. The instability of coagulant activity in the reaction mixtures containing placenta microsomes is unexplained at present. It may be due to some microsomal enzyme which degrades the coagulant activity, or possibly to some difference in the coagulant activity which develops in this system.

In the experiments with brain particles, from 76 to 100% of the original coagulant activity could be eluted from the particles with EDTA. In contrast, the activity detectable in the sediment was only 29 to 45% of that present in the EDTA washes. The reasons for this are not clear, but it was noted that the brain particles are difficult to disperse after centrifugation and possibly aggregates of particles coated with protein were present in the suspension of the final sediment, making less coagulant activity available in this system. Brain particles bind some protein nonspecifically and that may be the cause of the increased "stickiness" of the sediment. However, the brain particles do not bind unactivated Factor X. Binding of activated Factor X has been shown for lung (1) and placenta microsomes, human brain particles prepared by the present method, and NaCl extracts of human (3) and rabbit (4) brain. Calcium is required for the binding in these systems. The binding of activated Factor X by tissue extracts thus appears to be a point of basic similarity in the mechanism of action of tissue extracts from different sources.

The intrinsic phospholipid of placenta microsomes and brain particles can function as phospholipid in blood coagulation, as was previously shown for lung microsomes, although added phospholipid is required in the assay system employing low concentrations of placenta microsomes or brain particles. It should be noted that several years ago Ferguson (5) observed that tissue thromboplastin and phospholipid could act synergistically to enhance prothrombin conversion.

The placenta microsomes and brain particles appear to function as enzyme in the system used in these studies. Nemerson and Spaet (4) have recently reported the isolation from rabbit brain of a protein which is able to activate Factor X enzymatically. This material was thought to be the protein moiety of the brain thromboplastin. Their work supports the concept of the enzymatic function of tissue preparations in blood coagulation. However, as is reported in the accompanying paper (6), lung microsomes react very rapidly with calcium and a plasma protein believed to be coagulation Factor VII to yield a complex which acts as enzyme to activate Factor X. The product responsible for the coagulant activity detected in the present experiments appears to be Factor X, as determined by the experiments with Russell's viper venom. No studies have been done with placenta microsomes in the system employing purified Factor VII, but brain particles appear to function in the same manner as lung microsomes. Thus, the apparent enzymatic activity of the brain particles in activating Factor X in these experiments is probably due to a complex involving calcium and Factor VII rather than to the brain particles alone.

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REFERENCES

The Activity of Human Placenta Microsomes and Brain Particles in Blood Coagulation
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