PARTIAL PURIFICATION OF THE VASOCONSTRICTOR IN BEEF SERUM

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The demonstration that plasma from persons with hypertension is more vasoconstrictor than normal plasma would be direct evidence supporting a humoral mechanism for the disease. This necessitates evaluating effects of vasoactive substances which may be newly formed in the blood subsequent to withdrawal of the sample. The chief, and perhaps only, substance of importance in this regard is the vasoconstrictor appearing during the clotting or defibrination of blood.

Mainly with the purpose of removing this obstacle to the study of differences between plasmas from hypertensive and normal subjects, attention has been directed to the isolation and identification of the substance in serum responsible for its vasoconstrictor activity as measured in the vessels of the isolated rabbit ear. There are other reasons for establishing the identity of this substance, among which may be mentioned its probable rôles in hemostasis and intravascular clotting.

Since the problem of the serum vasoconstrictor is well over 50 years old, an extensive literature dealing with the subject is available (1-5) which will not be reviewed again here. Most of the papers discuss the pharmacological properties of the unpurified substance, its relative stability, and solubility. The results published show a high degree of inconsistency, reflecting the inability of previous workers to obtain quantitative information from their assay methods.

This report summarizes the results of work on the purification and properties of the serum vasoconstrictor in quantitative terms. The method of assay, consisting of perfusion of the blood vessels of isolated rabbit ears (6, 7), has proved capable of giving reproducible results within 10 to 20 per cent in the same preparation, with checks over several months within 25 to 50 per cent. A five-step purification procedure has been developed which yields a preparation of the vasoconstrictor material about 200 times as active, on a dry weight basis, as that in serum itself, with recovery of 25 to 50 per cent of the original activity in serum. The dry preparation can be stored without loss of activity, apparently indefinitely. At every stage of

* A preliminary report of this work has been published (Federation Proc., 6, 184 (1947)). The expense of this work was defrayed in part by a grant from the United States Public Health Service, Committee on Cardiovascular Disease.
the purification before the fifth, considerable instability of the substance is observed.

The method of purification described in detail below represents a summary of experience obtained with more than 60 runs, involving 900 liters of serum collected from almost 2 tons of beef blood.

The advantages of the procedure are that it is rapid, inexpensive, and gives preparations with reasonably constant properties. It permits purification without serious losses and provides a stable preparation suitable for accumulation for further purification. The procedure is adaptable to large quantities of serum without elaborate equipment.

EXPERIMENTAL

Collection of Serum—Clotted whole beef blood, obtained from the slaughter-house in a lot of 45 liters (eight buckets), is placed in a cold room as soon as received. The eight clots yield 15 to 20 liters of serum, of which 15 liters are processed at a time. The fluid is centrifuged in the cold. The collection of 15 liters of serum takes 2 days.

Step 1. Precipitation of Proteins—The proteins are precipitated from the serum, after adjusting the pH to 4.5 to 5.0, by means of 95 per cent ethyl alcohol. The pH adjustment has been found necessary for two reasons. First and most important is that large losses of activity result from concentration of solutions above pH 7. Second, more effective removal of proteins is obtained at the lower pH. The 15 liters of serum are processed in three 5 liter batches at this step.

5 liters of serum are adjusted to pH 4.5 to 5.0 with 130 ml. of 2 N HCl. After the addition of 6.7 liters of 95 per cent ethyl alcohol, the mixture is stirred well and left in the cold room overnight. The protein precipitate is removed by suction filtration, giving 9 to 9.5 liters of filtrate.

The combined filtrates (27 to 28 liters) are then concentrated at reduced pressure under nitrogen to about 300 ml., the temperature being kept below 40°. With the equipment at our disposal, this operation required 20 to 25 hours. Losses of activity are found to be increased with increase in distillation time. To control foaming, 10 ml. portions of 2-ethylhexanol are added as required.

Step 2. Removal of Acetone-Insoluble Impurities—To the 300 ml. of concentrate, 10 volumes of acetone are added. The mixture is shaken, then

1 The serum is most conveniently collected by means of an apparatus which permits the eight clots to drain simultaneously. This apparatus consists of a 24 gallon can, the bottom of which has been removed. The can rests on a board with a circular opening covered by \( \frac{1}{2} \) inch rectangular mesh galvanized screen, in turn covered by gauze. The expressed fluid falls into the can cover which rests on top of a 9 liter glass jar. The cover has two small holes drilled through its center.
stirred mechanically at room temperature until the insoluble phase becomes solid. This takes 30 to 60 minutes. The mixture is then chilled in the cold room overnight.

The clear, light yellow supernatant solution, which contains about 90 per cent of the active principle, is decanted from the solid (consisting mainly of inorganic salts, phosphatides, carbohydrates, and amino acids) and evaporated under reduced pressure in a bath kept below 40° to a volume of 80 to 100 ml. The troublesome foaming which occurs as the last of the acetone evaporates is controlled by increasing the pressure temporarily.

**Step 3. Extraction of Inactive Substances with Chloroform**—The mixture, which consists at this point of a yellow emulsion with floating dark brown oil, is transferred with the aid of an equal volume of chloroform and a few ml. of ethanol to a 500 ml. separatory funnel.

From here on, all operations are carried out in a cold room at 5°.

Shaking the two phases results in a stiff emulsion, which occasionally breaks overnight. More often it is necessary to add 10 gm. of solid ammonium sulfate and several ml. of methanol. After standing overnight, this results in a sharp separation of the phases into an upper, clear, light yellow aqueous layer, and a lower, brown chloroform layer. It has rarely been found necessary to resort to centrifugation.

The active principle is entirely in the aqueous phase, although the presence of some active material in the chloroform may be masked by the fact that the other substances in this layer are intensely vasodilator when tested in the rabbit ear.

**Step 4. Extraction of Active Principle with Butanol**—The aqueous layer is adjusted to pH 5.9 ± 0.3 with 5 N NaOH, 5 to 6 ml. being required. This adjustment is not critical. The solution is then saturated with ammonium sulfate by the addition of 0.6 gm. of the salt per ml., and subsequently extracted three times by shaking several minutes with equal volumes of butanol. The combined butanol extracts are left overnight at −10° and then decanted.

**Step 5. Precipitation of Active Principle with Dilituric Acid**—To the butanol extract, 150 ml. of a solution of dilituric acid (5-nitrobarbituric acid) has gone into the chloroform phase. This has been attended by a concomitant large increase in the volume of the chloroform layer with corresponding decrease in the volume of the aqueous layer. The effect is attributed to a change in the solvent properties of the chloroform resulting from the large quantity of dissolved material. Increasing the aqueous volume of the initial extraction or employing an additional aqueous extraction of the chloroform corrected this aberration.

The aqueous phase, which is at pH 2.9 to 3.2, has a concentration of activity 50 to 100 times greater than that of serum. The active material is not stable in this solution, over 55 per cent having been observed to disappear in 10 days at 5°.
Vasoconstrictor in Serum

Acid) in methanol (prepared by dissolving 12 gm. of the acid in 800 ml. of absolute methanol at the boiling point, cooling, and filtering) are added, resulting in an immediate precipitation of colorless solid.

After the mixture has stood overnight at 5°, a sample of the supernatant solution is tested for completeness of precipitation by the addition of a small quantity of the methanolic solution of the acid. If more precipitate forms, another 10 ml. of the diluteuric acid solution are added to the mixture. The procedure is repeated until complete precipitation is obtained, which is usually indicated by a faint yellow color.

The precipitate is collected by centrifugation and is then washed three times with small quantities of cold methanol to remove excess diluturic acid and butanol. The almost colorless solid is then dried in a desiccator by prolonged evacuation. The precipitate contains over 95 per cent of the activity present in the butyl alcohol extract. The yield from 15 liters of serum is about 2 gm. of this dry precipitate, containing 25 to 50 per cent of the activity found in the original serum.

Method of Assay—The assay is made by injection into the vessels of the isolated rabbit ear prepared as described by Page (6, 7). Solutions of the unknown are diluted until 0.2 ml. gives a response identical with that of 0.2 ml. of a standard control solution of the vasoconstrictor substance. The standard is a solution of material from Step 5 pooled from ten runs. A concentration of 1.2 γ per ml. has been arbitrarily designated as containing 1 unit of activity. On this basis, the rabbit ear preparation shows the responses to other vasoactive substances recorded in Table I. The reproducibility of the ear preparation responses has been found to be 10 to 20 per cent. The greater range shown for some of the substances in Table I is a result of the inability to obtain constrictions with them identical with those of serum vasoconstrictor. The composition of the perfusion fluid in gm. per liter is as follows: NaCl 8.2, KCl 0.84, MgCl₂·6H₂O 0.06, CaCl₂·2H₂O 0.04, glucose 1.0, NaHCO₃ 0.40. In addition, 8 ml. of 1 M K₂HPO₄ and 2 ml. of 1 M KH₂PO₄ are added per liter.

Properties of Purified Preparation—The range of activity of the purified preparation is 500 to 1000 units per mg. The bulk of the solid is composed of ammonium diluturate; analysis by direct nesslerization shows 60 to 85 γ of NH₃ per mg. The results of other color tests on this material are shown in Table II.

The heat stability of the substance in solution appears to be considerably increased by the fifth stage in purification. For example, neither heating at 100° for 5 hours at a concentration of 1 mg. per ml. nor standing for 1 month at room temperature at this concentration resulted in significant inactivation. Whether this increased stability is due to the stabilizing influence of the diluturic acid or to the removal of reactive impurities is not known.
The material does not show appreciable solubility in organic solvents. However, it can be dissolved in boiling water to the extent of 8 mg. per ml., but readily crystallizes on cooling. The solubility at room temperature is

**Table I**

*Activity of Several Vasoconstrictor Substances in Perfused Ear Vessel Preparation, Based on Arbitrarily Defined Unit of Activity*

<table>
<thead>
<tr>
<th>Substance</th>
<th>Units per mg. at 1 mg. per ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epinephrine hydrochloride</td>
<td>50,000–100,000</td>
</tr>
<tr>
<td>Tyramine</td>
<td>500–1,000</td>
</tr>
<tr>
<td>Histamine phosphate</td>
<td>3,000–4,000</td>
</tr>
<tr>
<td>Tryptamine hydrochloride</td>
<td>5,000</td>
</tr>
</tbody>
</table>

**Table II**

*Color Reactions of Purified Material of Step 5*

<table>
<thead>
<tr>
<th>Color reaction</th>
<th>Standard*</th>
<th>Comparable color obtained per mg. ppt. from Step 5</th>
<th>γ of standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gerngross-Voss-Herfeld for ( p )-hydroxyphenyl groups</td>
<td>Tyrosine</td>
<td>2–3</td>
<td></td>
</tr>
<tr>
<td>Pauly diazo for imidazoles</td>
<td>Histidine</td>
<td>2–3</td>
<td></td>
</tr>
<tr>
<td>Sakaguchi for guanidino groups</td>
<td>Arginine</td>
<td>&lt;2</td>
<td></td>
</tr>
<tr>
<td>Folin-Ciocalteu for phenols, indoles, etc.</td>
<td>Tyrosine</td>
<td>16–21</td>
<td></td>
</tr>
<tr>
<td>Ferric chloride</td>
<td>Epinephrine</td>
<td>&lt;2</td>
<td></td>
</tr>
</tbody>
</table>

* The quantity of standard substance used for color development is 10 γ for all reactions except the Gerngross-Voss-Herfeld reaction which requires 50 γ.

**Table III**

*Dialysis of Serum Vasoconstrictor Against 2 Volumes of 0.01 M Phosphate Buffer, pH 7.4, at 5°, without Stirring*

<table>
<thead>
<tr>
<th>Time</th>
<th>Concentration inside casing</th>
<th>Concentration outside casing</th>
</tr>
</thead>
<tbody>
<tr>
<td>hrs.</td>
<td>units per ml.</td>
<td>units per ml.</td>
</tr>
<tr>
<td>0</td>
<td>600</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>300</td>
<td>150</td>
</tr>
<tr>
<td>48</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>48; buffer solution replaced after 24 hrs.</td>
<td>125</td>
<td>75</td>
</tr>
</tbody>
</table>

about 2 mg. per ml.; at 5°, slightly more than 1 mg. per ml. Repeated recrystallization from hot water can be effected without any appreciable change in the activity of the precipitate. High concentrations of activity
In aqueous solution are easily obtained by removing the dilituric acid as its insoluble magnesium salt (8) with MgCl₂ or MgSO₄. No loss of activity is encountered in this procedure, but the use of basic magnesium salts results in considerable inactivation (30 to 50 per cent).

Studies on the chemical inactivation will be reported in a subsequent publication.

Some Properties of Active Principle As Found in Serum—Variable effects have been observed with regard to stability of the substance in serum, but, in general, centrifuged serum loses about half its activity in 5 days at 5°.

Considerable disagreement exists concerning the dialyzability of the serum vasoconstrictor. Quantitative data measuring this property are presented in Table III. The results indicate that the substance not only di-

| Table IV
| Concentration and Recovery of Beef Serum Vasoconstrictor |
|-----------------|-----------------|
| Fraction        | Original serum  | Dry weight       |
|                 | units per l.    | units per mg.    |
| Serum           | 300,000         | 4                |
| Ethanol filtrate, Step 1 | 240,000         | 10               |
| Aqueous concentrate, 300 ml, Step 1 | 150,000         | 10               |
| Acetone supernatant, Step 2 | 130,000         | 90               |
| Chloroform-washed aqueous phase, Step 3 | 120,000         | 200              |
| Butanol extract, Step 4 | 100,000         |                  |
| Dilituric acid ppt., Step 5 | 100,000         | 750              |

alyzes very rapidly and completely, but that it is not appreciably adsorbed by serum proteins.

The range in activity found in beef serum is 100 to 600 units per ml.

DISCUSSION

A summary of the purification process with regard to concentration and recovery of activity is presented in Table IV.

The question of the presence of multiple substances being responsible for the vasoconstrictor effect of serum (2, 9) is not answered by this study. However, the uniformity of behavior of the active material throughout the purification procedure, as well as the high recovery of activity from serum, argues for the principal measured effect as being due to a single substance. Certain physical properties, such as dialysis, are also consistent with this view. As can be seen from Table III, the rate of dialysis is approximately the same, starting with fresh serum and with serum from which half the activity has already been removed by dialysis.

If the assumption is made that the effect is due to a single substance,
then the data presented in Tables I and II rule out epinephrine, tyramine, histamine, and tryptamine on the evidence of activity-color ratios. For example, the value for the activity of epinephrine is 50,000 to 100,000 units per mg. The comparative color given by epinephrine in the ferric chloride test is 1000 \( \gamma \) of standard per mg., since epinephrine is used as the color standard. The ratio of activity in units per mg. to color in micrograms of standard per mg. for this test is therefore 50 to 100 for epinephrine. For the purified material of Step 5, the ratio is \( >350 \). The fact that this activity-color ratio is much greater with the purified material than with epinephrine, coupled with the assumption that activity is due to a single substance, makes it impossible for epinephrine to be that substance.

A similar argument may be applied to the other compounds with the Folin-Ciocalteu reaction for tyramine and tryptamine and the Pauly reaction for histamine.

Further experiments aimed toward ultimate isolation and characterization of the active material are in progress.

The authors wish to thank Mr. John M. Means, Miss Martha Bender, and Miss Elizabeth Hunt for valuable assistance.

**SUMMARY**

A five-step purification is described which results in a 200-fold concentration, on a dry weight basis, of the serum vasoconstrictor, a substance which appears during the clotting or defibrination of blood. The recovery of activity is 25 to 50 per cent of that in the original serum. The principal advantage of this procedure is that it yields a preparation with enhanced stability, permitting accumulation for further purification.

**BIBLIOGRAPHY**

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