PRESSOR SUBSTANCES IN ARTERIAL HYPERTENSION

III. CHEMICAL STUDIES ON PHERENTASIN*

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In the first paper of this series (1) the presence of a material producing a prolonged pressor response in rats was demonstrated in crude non-protein extracts of arterial blood samples from hypertensive subjects. This study has been extended and confirmed and the pressor substance named pherentasin (2). The present report is concerned with further purification of this material and with methods of chemical inactivation giving clues to its identity. Pherentasin has been separated from an accompanying depressor substance and has been purified by means of ion-exchange resins and extractions with solvents to the point where its pressor activity by weight is approximately equivalent to that of artenol.

Experimental Methods and Results

Crude non-protein extracts of arterial blood were prepared according to procedures previously reported (1, 2). Two biological methods have been used to indicate the presence of pherentasin: assay for pressor effects in the rat and the mesoappendix test (3) which detects vasoexcitor and vasodepressor substances altering the reactivity of blood vessels to epinephrine. These procedures have been discussed and described in detail (2, 4). Pherentasin has been shown to be present in the blood of many hypertensive subjects but not of normotensive individuals. Therefore, all studies concerning this vasoactive material have been carried out on hypertensive blood extracts or combined pools thereof and controlled by similar studies on extracts and pools of normotensive blood.

Relation of Pherentasin to the Content of Amines.—"Amine" content of various crude extracts was determined by a modification of the method of Richter (5) in order to ascertain

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whether or not the presence of pherentasin could be correlated with a high concentration of amines. To 1 ml. of extract, equivalent to 20 ml. of original blood, were added 5 ml. of water, 1 ml. of 2 N sodium hydroxide, and 6 ml. of toluene (c.p.). The mixture was shaken in a 60 ml. separatory funnel with a rotary motion intermittently for a period of three-quarters of an hour. After allowing the two layers to separate, a 3 ml. aliquot of the toluene was pipetted into a dry colorimeter tube. 3 ml. of chloroform (A.R.) and 0.1 ml. of 2 per cent picric acid in chloroform were added to the sample. The mixture was mixed intimately and read in a Klett

<table>
<thead>
<tr>
<th>Non-color-producing compounds</th>
<th>Color-producing compounds</th>
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<tbody>
<tr>
<td>Methylamine</td>
<td>1 per cent Ammonium chloride (cloudy)</td>
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<tr>
<td>Ethylamine</td>
<td>Heptylamine</td>
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<tr>
<td>Histamine</td>
<td>Isoamylamine</td>
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<td>Putrescine</td>
<td>Re-Butylamine</td>
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<tr>
<td>2-Aminoresorcinol</td>
<td>Trimethylamine</td>
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<tr>
<td>o-Toluidine</td>
<td>Phenethylamine</td>
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<td>Tyramine</td>
<td>Benzodrine</td>
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<td>Arterenol</td>
<td>Benzodrex</td>
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<td>Epinephrine</td>
<td>Methyl benzodrex</td>
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<tr>
<td>Neosynephrine</td>
<td>Ephedrine</td>
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<tr>
<td>Paredrine</td>
<td>p-Toluidine (slight)</td>
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<tr>
<td>Propadrine</td>
<td>Tryptamine</td>
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<td>Cobebrin</td>
<td>Pyridine</td>
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<td>Urea</td>
<td>Piperidine</td>
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<td>Guanidine</td>
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<td>Uric acid</td>
<td>Mescaline</td>
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<td>Guanine</td>
<td>Nicotine</td>
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<td>Phenylenediamine</td>
<td>Quinidine</td>
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<tr>
<td>Nicotinamide</td>
<td>2-Amino-1-(3,4-dihydroxyphenyl) propane</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>2-Methylamino-1-(3,4-dihydroxyphenyl) propane</td>
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photometer at 420 μm within 15 minutes. A standard curve was obtained with varying concentrations of isoamylamine and the "amine" level expressed as γ of isoamylamine per 100 ml. original blood.

It was found previously that the "amine" levels of crude hypertensive extracts were usually higher than those of normotensive extracts (1, 2). The method, however, does not measure true amine levels: (a) simple amines (such as methylamine and ethylamine) do not produce a color; (b) phenolic amines are not extractable from alkaline solution; (c) other nitrogenous organic compounds are detected (Table I); and (d) various vasoactive amines give different color-concentration ratios (Fig. 1). Nevertheless, an elevated "amine" level was considered as a rough index of a disturbance in amino acid metabolism in which deamination was depressed.
Certain crude extracts of blood formed precipitates with picric acid. Pherentasin also formed an insoluble complex with picric acid in alkaline solution which could then be hydro-

![Graph](image_url)

**Fig. 1.** The colorimetric determination of several known amines by a modification of the method of Richter. *a,* isomylamine; *b,* β-methylphenylethylamine; *c,* phenethylamine; *d,* tryptamine; *e,* p-toluidine.

lyzed with acid and separated partially from a material causing a depressor effect in the assay preparations. Although the pressor activity of the extracts was increased after hydrolysis of the picrate, the depressor material was not completely removed and the "amine" content was much lower than that of the crude extract.
Adsorption on Ion-Exchange Resins.—Purification by means of ion-exchange resins was attempted. Two resins, among eight tested, were found to aid in the purification of the active principle, amberlite IR-100H and IR-4B. 5 gm. of resin was added to 25 ml. of extract at pH circa 5.0, the mixture shaken intermittently for 30 minutes and allowed to settle. The supernatant liquid was decanted and the resin washed once with 5 ml. of distilled water added to the supernatant fluid. This fraction was adjusted to the original volume and labeled A in the case of the anionic exchange resin IR-4B, and C in the case of the cationic exchange resin IR-100H. The material adsorbed on the anionic resin was then eluted overnight with 20 ml. of 5 per cent sodium carbonate and washed with the eluting solution until the original volume was reached. The combined eluate was labeled AE. A similar procedure was carried out with the cationic exchange resin, eluting with 5 per cent hydrochloric acid and labeling the eluate CE. The procedure as used did not give a clean cut separation of the vasoactive material, and owing to the small quantities available an exhaustive study of the optimum conditions was not possible.

An illustrative experiment of six performed on further fractionation of the active material is shown in Fig. 2. Crude extracts, which were active by both methods of bioassay, were separated by the resins into four fractions: A, AE, C, and CE. Picrates were made on all fractions, and the filtrate remaining after separation of the precipitated picrate was labeled “filtrate.” The picrates were hydrolyzed and after removal of picric acid, the fractions were labeled “picrate;” all fractions obtained were assayed for vasoactive material. The CE fraction contained the majority of the pressor activity; A uniformly contained most of the depressor material, while both were found in C and AE. Vasoexcitor material detected by the meso-
appendix test was found in all fractions save three of the filtrates. The "amine" level was lower in the more purified fractions including CE. Further purification of the pressor material confirmed this observation and, therefore, lent less significance to the presence of the picrate color as indicating pheomycin. In no case were pressor or vasoexcitor substances found in fractions of normotensive arterial (or venous) blood treated in exactly the same manner. Dialysis of the active extracts overnight against cold tap water resulted in complete loss of pressor activity.

Isolation of Depressor Substance.—Throughout the entire investigation, spectrograms were made on the extracts at wave lengths of 212 m\( \mu \) to 1000 m\( \mu \) using a Beckman DU spectrophotometer. The only discernible peak was found at approximately 270 m\( \mu \). On purification the peak disappeared from the pressor fraction, but became sharper and more discrete in the depressor fraction. The peak in the absorption spectrum of the depressor material became accentuated and appeared to approach a maximum at about 265 m\( \mu \), the curve corresponding to that of the spectrograms obtained with compounds containing the adenine structure (Fig. 3). A pharmacological examination of the action of compounds containing the adenine nucleus (adenine, adenosine, adenylic acid, adenosinediphosphate and -triphosphate) showed that they induced effects on the rat's blood pressure identical with that of the depressor material found in extracts of blood. In addition, adenosinetriphosphate caused a vasodepressor response in the mesoappendix test. Extracts of normotensive blood uniformly contained considerable quantities of the depressor fraction. Although the absorption spectra of the extracts have helped in the identification of the depressor material, they have yielded only negative evidence as concerns the identification of pheomycin.

Extraction into Organic Solvents.—Since the active pressor extracts gave "amine" levels higher than inactive samples, and one of the basic steps in this procedure involved extraction into an organic phase, the solubility of pheomycin in various organic solvents was examined. The organic solvents tested were benzene, toluene, chloroform, carbon tetrachloride, and ethyl acetate. From acid or neutral solutions, none of the organic liquids extracted the pressor material. From alkaline solutions, however, varying degrees of extraction could be obtained. The most satisfactory recovery was accomplished by alkalinizing the extract to pH 10 and extracting with an equal or double volume of chloroform. Pheomycin was then obtained by reextracting back into acid solution, or evaporating to dryness with a drop of hydrochloric acid and dissolving the residue in acidified water. Whole blood has been extracted with chloroform in an attempt to facilitate the isolation of pheomycin; however, insufficient data have accumulated to draw definite conclusions as to the efficacy of this direct procedure.

Procedure for Preparation of "Pure" Pheomycin.—The procedure finally adopted for preparation of the purest fractions thus far obtained combined several of the above chemical methods. The CE fraction, which contained most of the active material was extracted from alkaline solution into chloroform, dried, and dissolved in acidified water. The resulting solution was a water-clear extract calling forth a prolonged pressor response, free of depressor material, and with a solid content equivalent to 10 to 20 \( \gamma \) per liter of original blood (Fig. 4). The active crude extracts contained an average of 1 to 7 mg. of solids per liter of blood. Therefore, the steps described resulted in at least a hundredfold purification, assuming no loss in the procedure. The extracts, at this point, tended to be somewhat unstable in that on standing the pressor activity was lost and sometimes a depressor action appeared.

Chemical Inactivation of the Vasoactive Material.—A number of experiments were performed in order to gain knowledge of the chemical nature of pheomycin. Since the amount of material was very limited, chemical inactivation studies were done. The extracts of blood, in their most purified form, were neutralized to pH 7.4 and subjected to the chemical procedures described below. The end-products were assayed biologically for pheomycin by the pressor and the mesoappendix tests.
Fig. 3. Absorption spectra of fractions made to purify the depressor material in extracts of arterial blood. 1, crude extract; 2, C fraction—after absorption of most of the pressor material on an anionic exchange resin; 3, fraction obtained after extraction of the depressor material into organic solvents. Injection of each of these fractions into rats caused lowering of blood pressure and bradycardia. The reaction became more pronounced as the material was purified. Note that the more purified depressor material has a spectrum close to that of adenosine.

1. Action of Acids.—2 ml. of extract + 1 ml. 0.1 N HCl was heated in a boiling water bath for 2 hours, cooled, neutralized, and assayed. No loss of activity by either biological test developed, indicating the stability of pherentasin in acid solutions.
2. Action of Base.—2 ml. of extract + 1 ml. 0.1 N NaOH was heated in a boiling water bath for 2 hours, cooled, neutralized, and assayed. All vasoactivity was lost, indicating the instability of pherentasin in alkaline solutions.

3. Action of Nitrous Acid.—2 ml. of extract + 0.7 ml. 0.1 N KNO$_2$ + 1.3 ml. 0.1 N HCl was allowed to stand at room temperature for 24 hours. The excess nitrous acid was expelled by mild heating and suction. The solution was neutralized and assayed. All vasoactivity was lost, suggesting that an amino group necessary for activity had been present.

4. Action of Hydroxylamine.—2 ml. of extract + 1 ml. 0.1 N NH$_2$OH·HCl$^1$ + 0.5 ml. 0.1 N NaOH was allowed to stand at room temperature for 24 hours, neutralized, and assayed. The prolonged pressor and vasoexcitor activity was lost; the extracts produced transient pressor and vasoexcitor effects similar to those found after injection of the usual short acting pressor amines. The result suggests that a carbonyl group was necessary for prolonged activity.

Fig. 4. A schematic representation of the purification of pherentasin present in crude extracts of arterial hypertensive blood.

5. Action of Semicarbazide.—2 ml. of extract + 1 ml. 0.1 N H$_2$N·NCONH$_2$·HCl$^1$ + 0.75 ml. 0.1 N NaOH was allowed to stand at room temperature for 24 hours, neutralized, and assayed. The prolonged pressor effects disappeared; the extracts caused only transient pressor responses, again suggesting that a carbonyl group had been present.

6. Action of Ketene.—2 ml. of extract in acetic acid buffer was exposed to ketene (6) for 10 and 30 minutes. The solutions were then made alkaline, extracted into chloroform, acidified, and reextracted back into acidified water. They were neutralized and assayed. Most but not all of the prolonged pressor activity was lost in the sample exposed to ketene for 10 minutes; exposure for 30 minutes resulted in destruction of all activity, suggesting that an amino group had been present.

DISCUSSION

Pherentasin is a compound, or possibly a family of compounds, soluble in water, chloroform, and ethanol in concentrations up to 90 per cent. It is dialyzable and non-protein in nature. The initial extraction procedure was devised

$^1$Neither hydroxylamine hydrochloride nor semicarbazide hydrochloride in the concentration used produced any effect on the assay systems.
with the following assumptions made: (a) arterial blood would yield more of the material than venous blood; (b) the substance was present in only minute quantities; (c) the material was unstable, being easily oxidized or inactivated; (d) it was amine-like in nature. In accordance with these postulates the crude extracts were prepared by drawing arterial blood directly into cold ethanol, removing the precipitated proteins, and acidifying the filtrate. The filtrate was concentrated, washed with ethanol, and freed of lipids by extraction with petroleum ether. Continuing with these assumptions, we have been able to form picrate complexes, extract pherentasin from alkaline solutions with organic solvents, and absorb it on cationic exchange resin.

During the early part of this investigation “amine” levels were determined as routine on crude extracts; it was found that those from hypertensive blood which contained pherentasin usually had higher values than those from normal controls (2). As the various steps in the present purification procedure were evolved, however, the “amine” level did not parallel closely the amount of either pressor or depressor material. It was, therefore, discarded as a chemical method for estimating the degree of purification of the pressor substance. Despite the fact that the “amine” level does not seem to be correlated with the presence of pherentasin, it possibly is important in hypertension and in other conditions as a measure of aberrated amino acid metabolism. The ultimate usefulness and significance of the level of amines in biological fluids depends on the elucidation of sensitive specific methods of analysis.

The fact that in the determination of the “amine” level the color-producing constituents could be extracted from alkaline solution into an organic solvent proved to be of value in the purification of pherentasin. It was found that pherentasin could be extracted by chloroform only from alkaline solutions; when the reaction of the solution to be extracted was either acid or neutral, no prolonged pressor activity was found in the organic phase. This observation definitely pointed to the fact that the material in question was an organic base of small molecular size. Further support of this hypothesis was offered by the property of dialyzability and solubility in high concentrations of alcohol and also by the fact that the pressor material was absorbed onto a cationic exchange resin. Purification of about thirtyfold was accomplished by the absorption of pherentasin on the resin and its elution therefrom.

Numerous reports have shown that groups of compounds and even specific members of a group have been successfully separated by the use of ion-exchange resins (7). To obtain maximum efficiency with this technique a rather substantial amount of starting material is required, since it is necessary to ascertain the most effective resin and the optimum conditions for absorption and elution (such as pH, time of absorption, ratio of resin to fluid volume, and amount of agitation). Owing to the limited quantity of pressor substance available only rough separations could be expected. The pressor material was found
mainly in one fraction, although it appeared in smaller amounts in others. Utilization of the resins resulted in fairly discrete separation of pressor and depressor materials. The use of ultraviolet absorption spectra and extraction with specific solvents have given presumptive evidence that the depressor material contains the adenine nucleus and is in all probability identical with adenylic acid, possibly derived from adenosinetriphosphate.

Further knowledge as to the structure of pherentasin was obtained by chemical inactivation of the vasoactive material. The pressor substance was stable in acid and unstable in alkaline solutions. The activity was lost on treatment with nitrous acid and prolonged ketenization. These two tests indicate that an amino group is essential for activity. On treatment with ketene for a relatively short period, and reaction with hydroxylamine and semicarbazide, the activity of the extract became markedly lessened. These results suggest that an active carbonyl group was concerned in the prolonged pressor activity. The extracts which were partially inactivated by hydroxylamine and semicarbazide also showed a change in biological response; instead of the prolonged pressor effect characteristic of pherentasin, a transient rise in blood pressure typical of that produced by the usual vasoactive amines was obtained.

One experiment has been performed in which the active material from a large quantity of blood was highly concentrated. Separation into two phases, an oily liquid and distinct crystals, was accomplished. Numerous recrystallizations were made. The oily liquid appeared to be soluble in acetone and contained substances which were depressor in the rat. The crystals, after washing with acetone, contained only pressor material.

One- and two-dimensional paper chromatography with ninhydrin as a developing reagent has given additional information (8). After further purification two distinct spots have been found and at least one seems to be associated with the prolonged pressor activity. Since under special conditions primary amines and pherentasin give a color with ninhydrin, the amino group essential to activity is probably a primary amine. Further characterization is in progress.

SUMMARY

Pherentasin, a material producing a prolonged pressor response in rats, has been procured from the arterial blood of hypertensive patients and purified. Pherentasin is of small molecular size, non-protein in nature, dialyzable, soluble in water and 90 per cent ethanol, and extractable into organic solvents from alkaline solution. It contains an amino group essential to activity, which is probably primary, and also contains an active carbonyl group. It has been found in concentrations up to approximately 20 μg per liter of blood.

BIBLIOGRAPHY