PRESSOR SUBSTANCES IN ARTERIAL HYPERTENSION

V. Chemical and Pharmacological Characteristics of Pherentasin*

By HENRY A. SCHROEDER, M.D., H. MITCHELL PERRY, Jr., M.D., EVIE G. DENNIS, and LAURA E. MAHONEY

(From the Hypertension Division, Department of Internal Medicine, Washington University School of Medicine, and Barnes Hospital, St. Louis)

(Received for publication, April 27, 1955)

A substance which is prolongedly pressor in the hypertensive rat has been repeatedly obtained from human hypertensive arterial blood but not usually from normotensive blood (1–9). This pressor substance has been called "pherentasin." Various inactivation procedures have indicted that it is a dialyzable primary amine, apparently non-phenolic, which is inactivated by nitrous acid, ketene, alkali and monamine oxidase (10), and that it might contain a carbonyl group. The substance exhibits a high pressor potency and only small amounts can be obtained from large quantities of arterial blood. The irregularities of the rat assay method which uses most of that obtained from a single patient for detection of its presence has made accumulation of sufficient quantities for direct chemical analysis difficult if not impossible. A better method of assay was therefore desirable.

This communication describes the results obtained with a new assay method which detects small quantities of a constrictor substance believed to be identical with pherentasin. Its pharmacology, chemical stability, and resistance or susceptibility to inactivation are discussed in an attempt to show what known vasoactive substances are not pherentasin and to throw some new light upon its chemical nature. The structure of the material has decided clinical importance in view of the newer successful therapeutic methods employed in controlling human hypertension (11).

Method and Materials

The assay method chosen was that of Furchgott (12). Briefly, the aorta from a freshly killed rabbit was cut spirally into a long strip and suspended in a bath containing 20 ml. oxygenated Krebs-Ringer solution maintained constantly at 37°C. by a thermostatically controlled water bath. A simple weighted lever and ink writer recorded contractions on a slowly revolving drum. 2 to 3 hours were required for a fresh strip to relax to a steady state. Before substances were tested, 0.2 ml. of 0.001 M ethylenediaminetetraacetate was added to

* Work done under a grant-in-aid from the National Heart Institute, United States Public Health Service, and the Lasdon Foundation.

the bath in order to chelate trace metals (especially copper) in the water which catalyze the oxidation of norepinephrine and other substances. Since the aortic strip was relaxed at the outset of each experiment, further lengthening did not usually occur unless the preparation was killed; constriction (shortening) of the vascular smooth muscle was therefore the criterion of activity of a test substance and relaxation after constriction that of an antagonist. The preparation remained active for 8 to 12 hours. Each test was done in quadruplicate, four preparations from the same rabbit being run simultaneously. As repeated washing of the bath removed most active substances from the muscle (save dibenamine), several tests were done on the same preparation. All strips were tested for contractility at the beginning and end of the experiment with norepinephrine in a final concentration of 10^{-8} (2.0 γ in 20 ml.). When the effects of known compounds were being evaluated, each was tested separately on the system and upon its response to norepinephrine.

Three pooled collections of blood drawn directly into alcohol were processed for pherentasin as previously described (3), being evaporated in a 60 gallon still by Monsanto Chemical Company and purified on ion exchange resins (5). The active extract from 17.54 liters hypertensive arterial blood was concentrated 15 times that of the original blood in isopropanol without drying at any of the 15 steps of the processing. A second, from 16.8 liters hypertensive arterial blood, was dried (step 16) and dissolved in dilute hydrochloric acid at a concentration 100 times that of the original blood. A third, from 16.0 liters normotensive venous blood was concentrated 400 times without drying at step 16 of the processing. Standard methods in use for 10 years were followed (4).

Although venous blood has been shown to contain less of the active pherentasin than arterial blood (3), the sensitivity of the rabbit aortic strip permitted an assay of small samples from hypertensive patients. Therefore, 20 to 30 ml. of venous blood was drawn directly into six volumes of cold ethanol, filtered, acidified, evaporated *in vacuo* to a concentration of twice the original one, refiltered and stored in the deep freeze before testing. In a few instances heparinized blood was tested directly, but foaming in the oxygenated bath made the preparation messy.

Concentrations of drugs are given as that concentration finally obtained in the bath according to weight. Concentrations of simpler substances are given as that concentration finally attained in terms of molarity. The active pooled extract was always tested in 1.0 ml. amounts, the equivalent of 15 ml. of whole blood, while venous blood extracts in 1.0 ml. amounts were the equivalent of 2.0 ml. of original blood.

Patients were divided into two groups, those receiving hydralazine and those receiving ganglionic blocking agents, reserpine, or no medication. This differentiation was important in view of the reported inactivation of pherentasin in the anesthetized rat by hydralazine (13). Normotensive subjects were hospital personnel and medical students. In an evaluation of venous blood extracts, reactions of the strip were graded as negative (0 to 1 mm. rise on the paper), slight (1 to 3 mm. rise), moderate (3 to 5 mm. rise), marked (5 to 25 mm. rise). Tests made in the whole anesthetized rat followed the routine method previously described (3).

Results on Various Samples

The active principle caused a slowly developing, prolonged contraction of the aortic muscle, which reached its height in 15 to 70 minutes and persisted until the strip was repeatedly washed with fresh solution, after which a slow recovery occurred. This type of response is termed the "slow reaction." Norepinephrine on the other hand caused a rapid contraction, reaching its height in 5 to 8 minutes and persisting until washing quickly reversed it. This type of

response is called the "rapid reaction"; it was produced by most known primary amines and by potassium (Fig. 1).

Venous Blood.—Untreated venous blood (1.0 ml.) was applied to a strip as soon as possible after drawing. Four samples from normotensive subjects gave no reaction. One caused a rapid reaction identical to that of epinephrine or norepinephrine; considerable pain was caused in drawing this sample because of small veins; the subject complained of palpitation and showed tachycardia, a transiently elevated systolic pressure, circumoral pallor, and sweating. Alcoholic extracts of the sample were inactive. Blood from two untreated hyper-

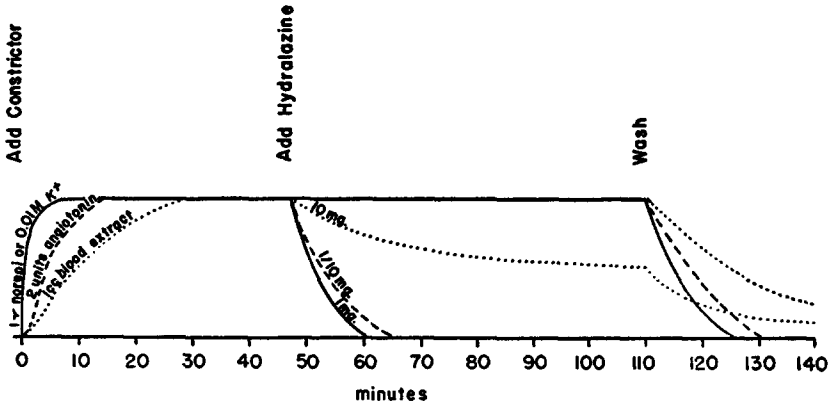

FIG. 1. Diagrammatic representation of rates of reaction of rabbit's aortic strip to norepinephrine and potassium ion, angiotonin, and active extract of hypertensive arterial blood traced from representative curves. The effect of hydralazine and of repeated washing of the strip with Krebs-Ringer solution is shown. Note the slow contraction and relaxation produced by pherentasin in the extract. Large quantities of hydralazine were necessary to depress the reaction after it had occurred, although inhibition was produced by mixing the two substances before testing.

tensive patients caused slow reactions while that from two did not. Activity was lost on standing overnight in the cold.

Venous Blood Extracts.—Alcoholic acidified extracts were obtained from 18 normotensive subjects: 14 were totally inactive, 3 showed slight activity, and one moderate constrictive effects. This last was obtained from a resident physician who had a record of mild intermittent hypertension disclosed to us after the results of the test were known. Extracts from 28 untreated hypertensive patients of all degrees of severity showed marked slow reactions in 8, moderate in 1, slight in 6, and no reaction in 13 cases. The negative reactions were given by extracts from patients with milder stages of the disease. Extracts from 15 other patients treated with hydralazine gave marked reactions in 2, moderate in 2, and none in 11.

Arterial Blood Extracts (Pherentasin) Pooled.—The first (hypertensive)

acidified isopropanol extract always caused a marked slow reaction in all strips tested (approximately 400). Acidified isopropanol itself gave no demonstrable reaction and the pH of the buffered Krebs-Ringer solution was unaffected. The second (hypertensive but dried) and third (normotensive) extracts were inactive in similar concentrations.

Effect of Hydralazine on Activity.—Three patients in malignant stages gave venous blood extracts which were strongly positive. After control of the hypertension by treatment with ganglionic blocking agents and hydralazine (10, 11) for 10 to 14 days, their normotensive blood extracts no longer showed constrictor activity.

Pharmacological Inactivation[1]

The Active Pool.—Attempts were made to ascertain whether or not the active material resembled known primary amines or other constrictor substances by observing the effect of mixing certain blocking agents with the material both before and after it was put on the strip.

(*a*) Regitine (2[N-p'-tolyl-N-(m'-hydroxyphenyl)-aminomethyl]-imidazoline) in final concentrations of 10^{-6} to 10^{-8} by weight blocks and reverses the actions of norepinephrine, epinephrine, and tyramine. Little or no decrease in constriction was produced by this agent immediately after adding it to the bath; in 5 of 15 experiments 30 to 45 per cent depression occurred only after 30 to 60 minutes while in the remainder no late changes were seen.

(*b*) Dihydroergotamine (10^{-5}) blocks norepinephrine, epinephrine, serotonin, and tryptamine. No blockade occurred; in fact, constriction was slightly enhanced.

(*c*) Iproniazid (isonicotinyl isopropyl hydrazide) (10^{-5}) enhances the constrictor action of tyramine. Actually it slightly decreased constriction already caused by the active material.

(*d*) Cocaine (2×10^{-7}) enhances the activity of epinephrine. No effect on the active material was observed.

(*e*) Dibenamine (*N*,*N*-dibenzyl-β-chloroethylamine) (10^{-6} to 10^{-8}) prolongedly blocks the receptivity of the aortic strip to norepinephrine, epinephrine, serotonin, tryptamine, and isoamylamine and partly blocks the constrictor action of histamine. It caused no change in the activity of the material.

(*f*) Pyribenzamine (2-[benzyl(2-dimethylaminoethyl)amino]pyridine) (10^{-6}) blocks the activity of histamine. It did not affect that of the active material.

(*g*) Atropine (10^{-7}) had no effect on the activity of the material; at high concentrations (10^{-3}) it was slightly depressor.

(*h*) Hydralazine (1-hydrazinophthalazine) (2×10^{-5} to 5×10^{-5}) inactivated the material slowly after the reaction had developed. When put on the

[1] Many of the statements in this section on the effects of known drugs on primary amines were based on the work of Furchgott (12, 14, 19).

strip first, this drug (2.5×10^{-5}) prevented activity of the extract. It also inhibited histamine, tryptamine, tyramine, guanidine, and norepinephrine at much smaller concentrations. Guanidine (10^{-3}), for example, was completely and rapidly inactivated by 10^{-7} hydralazine.

Therefore, the total activity of the material was not due to any of the above primary amines in the free state, although their presence in combined form could not be excluded. Hydralazine was the only one of these drugs which appeared to inactivate the material.

Material in Venous Blood.—A few similar studies were made on active single samples, concentrated twice, obtained from hypertensive patients.

(*a*) Dihydroergotamine (10^{-5}) did not block the constrictor action of the simple extract of blood from a patient with malignant hypertension; actually moderate enhancement occurred in three of four experiments, similar to that observed with the active pool.

(*b*) Hydralazine (5×10^{-7}) completely blocked the active material in all ten bloods tested, whether mixed previously or added to the bath after constriction was maximal.

(*c*) Cocaine (10^{-7}) enhanced slightly the activity of three samples and did not affect that of four others.

(*d*) Pyribenzamine (10^{-6}) did not affect the activity of two samples.

Chemical Inactivation

Chemical Spot Tests.—Samples of the two hypertensive pooled extracts were dried *in vacuo*. The partly purified dried material, a grayish amorphous powder, contained solids amounting to 15 mg. per 100 ml. of original blood. Standard spot chemical analyses performed by Mr. John Cary showed the presence of an aliphatic NH_2 radical by two tests, and considerable sulfur, but no carbonyl, alcohol, hydroxyl, sulfhydryl, $C = S$ or phenol groups (Table I). Both batches yielded the same results.

On paper chromatograms made in butanol-water mixtures, all three extracts produced three identically colored ninhydrin-sensitive spots with the same *Rf* values of 0.2 or less. The active extract contained three others with values of 0.33, 0.42, and 0.52.[2] The inactive (hypertensive, dried) extract demonstrated one extra spot with an *Rf* of 0.27, while the third, (normotensive venous) showed five others, with values of 0.24, 0.29, 0.35, 0.42, and 0.74. Therefore, drying caused the disappearance of both activity and the more hydrophilic substances.

[2] Five of these six spots have been found in all active extracts, even after much greater purification than was done to the material here tested (5). Counter-current partition chromatography (200 plates), performed by Merck and Co., Inc., separated the ninhydrin-sensitive material into the first 20 plates but the five spots remained together. It is possible, therefore, that hydrolysis of a polypeptide occurred during paper chromatography.

Effect of Drying.—The dried sample was found to lack pressor effect; in fact, it was depressor in the rat and somewhat inhibitory of epinephrine on the aortic

TABLE I

Analytical and Inactivation Studies on Pherentasin

Procedure	Result or change in activity	Method or Remarks
*Spot Chemical Analyses**		*Two Batches Identical*
Liters blood/milliliter extract	1/10	
Dissolved solids	1.48 per cent	
RCH_2OH	0	Conversion to alkali xanthates
R_2CHOH	0	" " " "
C=O	0	Phenylhydrazine reaction
Reactive CH_2 or NH_2	+	Naphthoquinone test
Aliphatic RNH_2	+	Fluorescence with fluorescein chloride in ultraviolet light
Phenol	0	Millon's test
S	0.5 per cent	Lassaigne's test
C=S	0	Iodine-azide reaction producing N_2
CSH	0	" " " "
Physical Measures		
Drying	Inactive	Rapid oxidation in air
Heat at pH 8.8	Inactive	? Primary amine
Heat at pH 2.0	0	
Heat at pH 7.6	−50 per cent	Partial hydrolysis ?
Chemical Measures		
Nitrous acid	Inactive	Primary amine
Ninhydrin	Inactive	Primary terminal amine
Hydralazine	Inactive	Color formed
Semicarbazide	Rapid reaction	? Carbonyl linkage
Hydroxylamine	Rapid reaction	" "
Enzymatic Inactivation		
Amine oxidase (kidney)	Inactive	Primary terminal amine
Tyrosinase (potato)	0	No mono- or orthodihydroxyphenol available
Pepsin, trypsin, chymotrypsin, papain, protease, carboxypeptidase	0	
Papain + cysteine	Inactive	Aliphatic peptide linkage

* From Feigl, F., Qualitative Analysis by Spot Tests, Amsterdam, Elsevier Publishers, 3rd edition 1946. Present analyses performed by Mr. John Cary. The limits of determination with known compounds were 4 per cent for the alcohols and 1 per cent for the other tests.

strip. Therefore a portion of the active pool was dried, redissolved, and injected into three rats. Acute diastolic depression of 22 to 54 mm. Hg and prolonged (15 to 20 minutes) depression of 10 to 20 mm. Hg occurred. The originally active dried material from one patient and from the pool were both inactive

on the aortic strip. On the other hand, the undried active pool, boiled for 30 minutes in a water bath, was pressor in the same three rats and in another, with acute or prolonged systolic elevations of 10 to 40 mm. and diastolic of 7 to 30 mm. without being acutely depressor as is usual with unboiled crude extracts.

Effect of Alkali and Acid.—Heating at 100°C. for 30 minutes at pH 8.8 or greater destroyed the material when sodium hydroxide, borate, citrate, and carbonate were the alkalis. No change in activity was caused by heating at various pH's from 7 to 2.0. Heating with sodium acetate and disodium hydrogen phosphate at pH 7.6 diminished the activity by 40 and 50 per cent respectively. When high concentrations of potassium ion were used as the alkali, a rapid norepinephrine-like contraction occurred.

Effect of Inorganic Ions on the Test System.—Since dipotassium hydrogen phosphate caused constriction of the aortic strip, a number of simple inorganic ions were tested. Relatively high concentrations of sodium ion (0.07 to 0.1 M) added to the isotonic bath caused slight constriction when given as the chloride, borate, carbonate, and acetate; smaller concentrations (0.05 to 0.01 M) did not. Borate was slightly more active in this respect. Alteration of the pH of the bath to alkaline caused some constriction. Potassium chloride caused stepwise constriction of the "rapid" type as the concentration was increased from 0.02 to 0.1 M; dipotassium hydrogen phosphate was more potent, effects being considerable at 0.005 M and being marked at 0.05 M solutions. Disodium hydrogen phosphate was constrictor very slightly and transiently (0.05 M), trisodium phosphate (0.015 to 0.003) slightly more so; but these high concentrations changed the pH. The dichlorides of magnesium, iron, cobalt, chromium, zinc, copper, and mercury and the citrate of manganese had no demonstrable depressant effect upon the strip in concentrations of 0.0005 M.

The test system was therefore affected by high concentrations of potassium ion, by alkalinity, and by hypertonicity. It was not affected by phosphate nor by the other inorganic cations and anions tested.

Dissimilarity of the Active Material and Potassium Ion.—On the remote possibility that potassium ion in the active material might cause constriction of the strip, all pharmacological tests were performed on inactivated heated extracts made alkaline to pH 8.8 with dipotassium hydrogen phosphate in high concentrations. The potassium ion alone or in the mixture caused a typical rapid reaction as opposed to the slow reaction of the active principle. Dihydroergotamine markedly enhanced the reaction caused by potassium; atropine had no effect in high concentrations, and pyribenzamine and iproniazid caused slight enhancement; these reactions differed from those observed with the active material. It was probable, therefore, that activity was not caused solely by potassium nor by any of the other metal ions tested.

Effect of Heavy Metals.—In an attempt to demonstrate inactivation by che-

lation with a dipeptide (15, 16), samples of the active pool were mixed with various metal salts at concentrations of 0.01 M and allowed to stand. Significant depression of activity was caused immediately by manganous citrate, which abolished it completely on standing at room temperature for 24 hours. Adding manganous ion at the height of the reaction was also depressor. This effect could not be reversed by large concentrations of ethylenediaminetetra-

TABLE II

Inactivation of Pherentasin by Metal Ions and Metal-Binding Agents

Estimated activity, per cent of control values.

Substance	Immediate	4 to 5 hrs.	24 hrs.	Boiled after 24 hrs.	Color developed
Mg^{++}	91	130	61	0	0
Cr^{++}	187	180	*12*	0	0
Mn^{++}	*45*	*17*	0	—	Pink cloudy
Fe^{++}	70	134	58	0	0
Co^{++}	59	50	50	0	Faint pink
Cu^{++}	96	107	*27*	0	0
Zn^{++}	84	137	54	27	0
Hg^{++}	168	51	115	0	0
None	100	100	100	80	0
Hydralazine	*46*	*20* (2)*	0 (18)		Deep blue
NaSCN	100	0 (1½)			Yellow-orange
$Na_2Fe(CN)_5NO$	110	0 (2)			0
NaN_3	89	0 (2)			0
8-Hydroxyquinoline	*35*	0 (1)			Pale green
Na_2H_2EDTA	100	0 (3)			0
Cysteine	100		100 (120)		0

All metals were added to the active material in 0.01 M concentrations, giving a final concentration in the 20 ml. bath of 0.0005 M. The binding agents were added in 2 to 5 mg. amounts per ml. extract. None of the metal ions alone affected the test system at 0.0005 M concentrations. Figures in italics represent more than 50 per cent inhibition.

* The figures in parentheses indicate the number of hours of incubation at room temperature when different from that shown at the top of the column.

acetate. After 24 hours partial inactivation occurred with cupric and chromous chlorides, while little or none was produced by magnesium, ferrous, zinc, cobaltous, and mercuric chlorides (Table II). Boiling with the metal after standing 24 hours destroyed the material in all cases save with zinc. Manganous ion also rapidly inactivated guanidine and angiotonin.

Effect of Metal-Binding Agents.—Because hydralazine and certain other antihypertensive drugs have in common the ability to combine with some trace metals (13, 22), experiments were designed to ascertain the effect of these

binding and chelating agents upon the active principle. When 5 mg. of hydralazine was mixed at room temperature with 1.0 ml. of extract, 80 per cent of the activity was abolished in 2 hours and all in 18 hours, while untreated extract was not affected, even on standing for 5 days. A deep blue color developed. Iproniazid, however, failed to inactivate the material on standing or to produce a color. Smaller concentrations of sodium thiocyanate (2.0 mg.) completely inactivated the material after 90 minutes at room temperature, with the production of an orange-yellow color and 8-hydroxyquinoline (2.0 mg.) inactivated it in 60 minutes with the development of a light greenish color. Sodium nitroprusside (2.0 mg.) and sodium azide (2.0 mg.) likewise inactivated the material on standing for 2 hours without change in color. Disodium ethylenediaminetetraacetate (5.0 mg.) completely inactivated the substance on standing for 3 hours without change in color. Cysteine was ineffective and β-mercaptopropionic acid either failed to change activity in small concentrations or was toxic to the strip in larger amounts (Table II). When added to the muscle constricted by the active material, only 8-hydroxyquinoline slowly depressed the strip in a manner similar to that produced by hydralazine. The constricting effect of norepinephrine was abolished only by azide and nitroprusside of the six substances tested.

In view of the possibility that a metal caused the changes in color, ferric, zinc, cobaltous, cupric, vanadyl, and nickel salts in small concentrations were mixed with hydralazine, 8-hydroxyquinoline, thiocyanate, ethylenediaminetetraacetate, and nitroprusside. Colors were immediately produced by the ferric salt with the first three substances, which were identical to those developing slowly when the same binding agent was mixed with the extract; none of the other metals gave similar colors. The spectra of the ferric complexes and those of the extracts were not comparable when measured by the Beckman spectrophotometer.

Evidence for Primary Amine Configuration.—While chemical spot tests showed the presence of primary amine, confirmatory evidence was offered by inactivation of the material by nitrous acid and ninhydrin. In 30 minutes at room temperature, 0.018 M nitrous acid inhibited activity by one-half; doubling this concentration suppressed it completely. A relationship between the concentration of acid and inactivation was demonstrated over this interval of time, but in 4 hours 0.005 M acid destroyed the material. Ninhydrin (1 per cent) caused a yellow color to appear and inactivated the constrictor substance. A biuret reaction, however, was consistently negative.

Evidence for Carbonyl Group.—Hydralazine, a carbonyl as well as a metal-binding agent, was first used to treat hypertension because pherentasin apparently contained a carbonyl group essential to prolonged pressor activity (3) and was inactivated by the drug (13). This line of reasoning may have been erroneous, in view of the data already presented. Two other carbonyl

reagents were tested. Semicarbazide (also a hydrazide) which had converted the prolonged pressor response in the rat to a transient one (3) was mixed with the active extract. The reaction between them was apparently gradual and dependent partly upon pH; at 7.5 in 2 hours, the material still gave a slow reaction but at 18 hours a rapid reaction had developed and at 48 hours complete inactivation had occurred. At pH 4.0 the rapid action was present 72 hours after mixing. Hydroxylamine, on the other hand, enhanced the rate of reaction at acid pH considerably within 30 to 90 minutes of mixing but failed to affect it at neutral or slightly alkaline pH after 18 hours.

Dissimilarity of the Active Material and Angiotonin (Hypertensin).—The acute pressor action of angiotonin in the rat does not resemble the prolonged pressor action of pherentasin. On the aortic strip, however, 1 unit of angiotonin caused a typical slow reaction, but pharmacological and chemical tests differentiated the two substances. (*a*) About one-fiftieth of the amount of hydralazine necessary to inactivate equiconstrictor doses of the active extract completely abolished the action of angiotonin. (*b*) Regitine caused moderate inactivation. (*c*) Cobalt chloride and manganese citrate (0.01 M) both immediately inactivated angiotonin, unlike pherentasin. Similarities only appeared in the susceptibility of both substances to heat at an alkaline pH and stability in acid. It is likely that pherentasin was not angiotonin, although similar groups may have been present in the molecules of both.

Enzymatic Inactivation.—The alcohol-free constrictor material and the test system were not affected by papain, trypsin, chymotrypsin, protease, and pepsin. Papain and cysteine at pH 2 inactivated pherentasin partly and at pH 7 completely; cysteine alone had no effect. Renal amine oxidase was of itself slowly constrictor; therefore, semiquantitative evaluation was necessary. A tenfold concentration of oxygenated alcohol-free extract incubated with amine oxidase for 30 minutes at 20°C. caused no greater constriction of the strip than with the enzyme alone, while a mixture of the two applied at once was considerably more active. Potato tyrosinase also contained substances constrictor for the strip but did not inactivate the material, while carboxypeptidase was inert.

Some hydrolysis at pH 7.6 and 100°C. for 30 minutes was suggested by the observations that regitine and pyribenzamine then caused slight inactivation when added at the height of the reaction. Dihydroergotamine, previously inert, enhanced activity slightly. This change did not occur when the material was heated at pH 2.

DISCUSSION

The present series of experiments again demonstrates the presence of a vasoconstrictor substance in extracts of human hypertensive blood. The

substance in 2.25 mg. quantities[3] produces a prolonged, slowly developing constrictive action *in vitro* on the smooth muscle of the rabbit aorta, which disappears slowly after washing and is remarkably resistant to the usual pharmacological blocking or synergistic agents. From the behavior of known vasoactive materials on the aortic strip, we can state categorically that the constrictive action was not caused by the presence of free tyramine, norepinephrine, epinephrine, tryptamine, serotonin, histamine, isoamylamine, cholinergic agents or angiotonin. The large quantities of guanidine necessary to cause a slow reaction (10 to 20 mg.) and its extreme susceptibility to hydralazine (0.1 mg.) definitely exclude this substance as the sole active agent. The presence of one or more of these substances in a peptide linkage, however, could not be excluded. The failure of adrenolytic and sympatholytic substances to block the action of the vasoconstrictor substance places it in a class of compounds separate from the sympathomimetic amines and suggests that it acts directly upon smooth muscle and not on nervous end organs.

Failure to find activity in all extracts of hypertensive venous blood is not disturbing. Previous studies (2, 3) have shown that more is present in arterial blood, indicating that it may be bound to arterial smooth muscle at its site of action. Venous blood from patients with severe hypertension has usually been active, confirming our impression (5) that the amount present is roughly proportional to the severity of the hypertension in terms of the non-neurogenic or nephrogenic component (10). The similarity of the substance found in arterial blood extracts to that in venous blood extracts appears established by these experiments.

Some activity has occasionally been found in arterial blood from normotensive patients suffering from febrile illnesses and congestive heart failure (5) but none has been demonstrated in extracts from normal individuals (4). The normotensive venous pool, although inactive, does not constitute an exact control; difficulties in obtaining large quantities of arterial blood from normal subjects were insurmountable. This blood came from Kahn positive samples in a blood bank 2 days old and serves only to indicate that pherentasin is not a product of the processing of the extracts. Other studies have shown that it is confined to hypertensive blood.

Identity of the Active Substance with Pherentasin.—As originally described, pherentasin was detected by its prolonged pressor activity in the rat and its ability to sensitize the vessels of the rat mesoappendix to topically applied

[3] This material was quite crude (150 mg. dry weight per liter arterial blood). Previous intensive efforts to purify pherentasin have yielded approximately 0.02 mg. per liter of arterial blood (4), although losses may have been considerable. The present active material was not purified further as the assay method was highly sensitive. It is possible that the biuret test was negative because of the very small quantities present in this extract.

epinephrine (3). It is necessary to consider whether or not the present substance is indeed pherentasin as extracted previously (1–9). The similarities are as follows: (*a*) Methods of extraction were identical except for the quantities and were carried out by the same technicians. (*b*) The active pool here discussed caused a prolonged pressor response in rats. (*c*) Both were inactivated by nitrous acid. (*d*) Both were inactivated by hydralazine, although the ratio of susceptibility of the material to the drug was high in the rat and considerably lower on the aortic strip. (*e*) Hydroxylamine and semicarbazide altered its prolonged pressor activity in the intact rat to a short acting one (4) and changed it to a substance acting rapidly on the strip. (*f*) Both were stable to acid and unstable to alkali. (*g*) Both formed complexes with ninhydrin. (*h*) Ordinary blocking agents were inactive on both test systems. It is likely, therefore, that the substance constricting the rabbit's aortic strip contained the same substance described previously as pressor for the rat.

Chemical Nature.—Various hypothetical formulae have been proposed for pherentasin (4, 5, 10), all of them representing mere guesses. At least one primary amine group is present and necessary for activity; it appears to be terminal. Carbonyl may be present; these experiments do not exclude this possibility while previous ones point to it (4). Sulfur was unexpectedly found in large amounts, but it was not in any of the usual organic configurations in the *dried* (inactive) material and may have been a contaminant. Inactivation by manganese, but not by other metallic ions, suggests that a peptide linkage of a specific type or a formula $RCHNH_2CONH_2$ could have been present (15). Inactivation of the polypeptide angiotonin by cobalt ion, for example, is consistent with one of its known peptide linkages (15, 17). Destruction by papain, but not by other proteolytic enzymes, suggests an aliphatic structure in a peptide bond.

The susceptibility of pherentasin to inactivation by hydrazides is of little aid in determining chemical structure. Hydralazine enhances monamine oxidase in kidney (18) and iproniazid inhibits that in smooth muscle of aorta; the latter inhibition enhances the activity of tyramine (19). Because both hydrazides depressed pherentasin, their effect was probably not the result of action on this enzyme. Not too much is known of the mode of vascular action of the hydrazides. They are carbonyl reagents and bind certain trace metals (13). They act as antienzymes, for histaminase (20) and decarboxylase (18), and show considerable specificity. They are bound to protein and arterial mash. The slow reaction between hydralazine and pherentasin *in vitro* suggests a chemical combination of the active material and the reactive hydrazine group; the formation of a deep blue color may indicate complex formation. Hydralazine did not react directly with the primary amines tested (13), suggesting another type of linkage with pherentasin. On the other hand, some evidence has been presented for a direct chemical reaction with the smooth

muscle of the strip in a manner similar to that which dibenamine undergoes, since reaction was inhibited by prior application of the drug. Perhaps the action of hydralazine can best be explained by both types, direct chemical combination with pherentasin and direct binding to arterial smooth muscle.

Inactivation of pherentasin in time by six substances having in common the ability to bind metals suggests metal binding is related to inactivation. There are three possible explanations: (*a*) Iron was present as a contaminant and inactivation was not related thereto. (*b*) The reaction of the binding agents caused secondary inactivation of pherentasin by release of some unknown reactive substance previously complexed. (*c*) Iron is present and necessary for activity. The slow production of color changes by three of these substances, identical to those quickly formed with ferric ion, suggests that if iron is involved, it is quite tightly bound. Another trace metal, however, could be essential for activity.

Only a rough estimate can therefore be made of the structure of pherentasin, based on the available information. It contains a primary amine group on a terminal carbon atom, may have an aliphatic peptide linkage and one or more carbonyl groups essential to prolonged activity. Sulfur may be present in a configuration sensitive to rapid oxidation. A metal, possibly iron, may be bound to the molecule. In simplest terms, the active material may contain cysteine or a metabolic derivative and one or more amino acids with a terminal active amine. If so, a polypeptide altered by abnormal metabolism might be suspected. Under certain circumstances the crude extract has altered from a pressor to a depressor material, suggesting oxidation of an active group to an inactive form.

Present uncertainties as to structure are due largely to our inability to collect enough pure material for elementary chemical analysis. The substance, unknown as it is, provides, however, a logical method for basic evaluation of antihypertensive compounds not acting on autonomic nerves. In the study of these agents it was found that (*a*) Pherentasin *in vitro* was inactivated slowly by six drugs with antihypertensive actions in man or animals (22). (*b*) Pherentasin disappeared from blood when patients were treated successfully with hydralazine, a metal-binding agent which lowers blood pressure and may increase renal plasma flow (21) in man. (*c*) No inactivation was produced *in vitro* by substances acting on constrictor nerves. (*d*) The common chemical denominator of the substances destroying pherentasin was their ability to bind certain trace metals. (*e*) Pherentasin was no longer pressor in rats pretreated with hydralazine (13).

SUMMARY AND CONCLUSIONS

Some pharmacological and chemical qualities of pherentasin, a vasoconstrictor substance procured from human hypertensive blood, were studied by

a new assay method using the spirally cut rabbit aorta. Of a number of drugs tested, six metal-binding agents including hydralazine inactivated the active principle. The material was stable in acid but not in alkali. It was destroyed by drying. Chemical analysis and inactivation procedures suggested the presence of primary amine and considerable sulfur; a peptide linkage was suspected because of inactivation by manganous ion and papain. The material was remarkably resistant to most pharmacological agents and appeared to act directly on smooth muscle.

The authors are deeply appreciative of the help and advice of Dr. Robert F. Furchgott in setting up his method and in evaluation of pharmacological tests, of Dr. Dean F. Davies for drawing the blood and supervising the processing of the extracts and of Dr. F. C. Meyer of Monsanto Chemical Company for the use of facilities for vacuum distillation of the crude alcoholic extracts.

BIBLIOGRAPHY

1. Schroeder, H. A., and Stock, C. C., On the pressor activity of extracts of hypertensive blood, *J. Clin. Inv.* 1942, **21,** 627.
2. Stock, C. C., and Schroeder, H. A., Pressor substances in arterial hypertension: Activity and amine content of crude extracts of blood, *Am. J. Physiol.*, 1950, **160,** 409.
3. Schroeder, H. A., and Olsen, N. S., Pressor substances in arterial hypertension. II. Demonstration of pherentasin, a vasoactive material procured from blood, *J. Exp. Med.*, 1950, **92,** 545.
4. Olsen, N. S., and Schroeder, H. A., Pressor substances in arterial hypertension. III. Chemical studies on pherentasin, *J. Exp. Med.*, 1950, **92,** 561.
5. Davies, D. F., Olsen, N. S., and Schroeder, H. A., Pressor substances in arterial hypertension. IV. Quantitative and qualitative studies of pherentasin, *Circulation*, 1952, **5,** 380.
6. Schroeder, H. A., Goldman, M. L., and Olsen, N. S., Pressor substances in hypertensive blood, *Fed. Proc.*, 1948 **7,** 110.
7. Schroeder, H. A., Olsen, N. S., and Goldman, M. L., Pressor substances in human hypertensive blood, *in, Trans. 2nd. Conf. Factors Regulating Blood Pressure,* 1948, Josiah Macy, Jr. Foundation, 118.
8. Schroeder, H. A., Goldman, M. L., and Olsen, N. S., Pressor substances in extracts of hypertensive blood, *J. Clin. Inv.*, 1948, **27,** 555.
9. Schroeder, H. A., and Olsen, N. S., Pherentasin: A pressor substance present in arterial hypertension, *J. Clin. Inv.*, 1950, **29,** 844.
10. Schroeder, H. A., Hypertensive Diseases. Causes and Control, Philadelphia, Lea and Febiger, 1953.
11. Schroeder, H. A., Management of arterial hypertension, *Am. J. Med.*, 1954, **17,** 540.
12. Furchgott, R. F., and Bhadrakom, S., Reactions of strips of rabbit aorta to epinephrine, isopropylarterenol, sodium nitrite and other drugs, *J. Pharm. and Exp. Therap.*, 1953, **108,** 129.
13. Perry, H. M., Jr., and Schroeder, H. A., Studies on the control of hypertension

by hyphex. III. Pharmacological and chemical observations on 1-hydrazinophthalazine, *Am. J. Med. Sc.*, 1954, **228,** 396.

14. Furchgott, R. F., personal Communications.
15. Smith, E. L., Aspects of the specificity and mode of action of some peptidases, *in* Enzymes and Enzyme Systems (J. T. Edsall, editor), Cambridge, Harvard University Press, 1951, 47.
16. A Symposium on the Mechanism of Enzyme Action, (W. D. McElroy and B. Glass, editors), Baltimore, Johns Hopkins Press, 1954, 221.
17. Schales, O., Kidney Enzymes and Essential Hypertension, *Advances Enzymol.*, 1947, 513.
18. Perry, H. M., Jr., Teitlebaum, S., and Schwartz, P. L. Effects of antihypertensive agents on amino acid decarboxylation and amine oxidation, *Fed. Proc.*, 1955, **14,** 113.
19. Furchgott, R. F., Weinstein, P., Huebl, H., Bozong-Mehri, P., and Mensendiek, R. Effect of inhibition of monoamine oxidase on response of rabbit aortic strips to sympathomimetic amines, *Fed. Proc.*, 1955, **14,** 341.
20. Gross, F., Schuler, W., Tripod, J., and Meier, R., Hemmung der Diaminooxydase (Histaminase) durch Phthalazinderivate, *Experientia*, 1952, **8,** 229.
21. Schroeder, H. A., Effect of 1-hydrazinophthalazine in hypertension, *Circulation*, 1952, **5,** 28.
22. Schroeder, H. A., and Perry, H. M., Jr., Antihypertensive effects of metal binding agents, *J. Lab. and Clin. Med.*, in press.