# A CRYSTALLINE PRESSOR SUBSTANCE (ANGIOTONIN) RESULTING FROM THE REACTION BETWEEN RENIN AND RENIN-ACTIVATOR

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Plate 2

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Renin is a protein-like substance extractable from normal kidneys which, when injected intravenously into animals, causes prolonged rise in arterial blood pressure (Tigerstedt and Bergmann, 1898). When purified by the method of Helmer and Page (1939), it was found to be highly active when injected into intact animals, but produced no vasoconstriction when perfused with Ringer's solution through a dog's tail or rabbit's ear. Kohlstaedt, Page, and Helmer (1939) found that the pressor activity could be restored by addition of a protein-like substance contained in plasma and red blood cells. They designated this substance renin-activator to connote only that renin was inactive as a pressor substance without it. Indeed, there is some evidence, which will be presented in this communication, that it is the substrate on which renin acts. Since renin-activator restored the pressor activity of purified renin, it seemed reasonable to search for products resulting from the interaction of renin and renin-activator. A study of this problem has furnished the data of this paper. In short it was found that a pressor substance was produced which was heat-stable and which yielded crystalline derivatives. For this substance we suggest the name "angiotonin" [Greek ἀγγεῖον, blood vessel, + τόνος (τείνω), strain].

## *Methods*

Renin was prepared from pig's kidneys by the method of Helmer and Page (1939). Renin-activator was separated and concentrated by fractional precipitation of ox serum using potassium phosphate as precipitant.

Inactive substances were removed by adjusting the phosphate concentration of ox serum to 1.5 molar with 3 molar potassium phosphate. By raising the phosphate concentration to 2 molar, most of the active material was precipitated. This precipitate was dialyzed in cellophane sacs against running tap water. The precipitate which forms in the sac was discarded and the residual solution again precipitated between 1.5 and

2 molar potassium phosphate. The precipitate was dialyzed until free of potassium phosphate.

Renin-activator may also be prepared by fractional precipitation with ammonium sulfate, the active fraction being that precipitated between 0.3 and 0.55 per cent saturation at room temperature.

## *Preparation of Angiotonin*

*1. Heating Method.*—Renin-activator (200 cc.) was diluted with an equal volume of 0.9 per cent sodium chloride solution, 10 cc. of renin added, and the mixture incubated at 40°C. for 10 minutes. The reaction was halted by immersing the flask in boiling water for 5 minutes. The coagulum was thrown down in a centrifuge, the supernatant fluid decanted, and extracted twice with warm ethyl alcohol. The washings were combined with the supernatant fluid and evaporated to dryness under reduced pressure. The residue was extracted with anhydrous ethyl alcohol and the extract filtered through paper. The alcohol was evaporated in a current of air on a steam bath, the residue dissolved in water and extracted in a separatory funnel with ethyl ether. The ether dissolved in the aqueous phase was removed by heating on a steam bath. A few drops of glacial acetic acid were added to bring the pH of the solution to approximately 3.5, the solution was heated on the steam bath for 15 minutes and then placed in a refrigerator for 1 to 2 hours. A gum-like material formed which was filtered off. The filtrate was made up to 25 cc. with water. It has been found satisfactory, if desired, to stop the reaction between renin and activator by addition of 4 volumes of alcohol instead of heat.

The clear, colorless filtrate was further purified by precipitation with phosphotungstic acid and decomposition of the phosphotungstate with barium hydroxide. The barium phosphotungstate was removed by centrifuging and the excess barium by addition of sulfuric acid.

*2. Ultrafiltration Method.*—Activator and renin in the proportion of 20:1 were mixed in the cup of an ultrafilter fitted with a 4 per cent collodion membrane. Filtration proceeded by suction at room temperature for 15 minutes and for 45 minutes in the refrigerator. Angiotonin passed through the membrane while renin and activator were held back.

*Preparation of Crystalline Picrate and Oxalate (Figs. 1 and 2).*—The picrate was prepared by addition of saturated alcoholic solution of picric acid to a concentrated alcoholic solution of angiotonin, and then ether was added until no further precipitate formed. On standing overnight in the refrigerator the picrate settled to the bottom of the flask. The precipitate was washed several times with ether, dissolved in hot absolute ethyl alcohol, and filtered. The filtrate was concentrated to a small volume and allowed to stand overnight in the refrigerator when a crystalline picrate of high pressor activity settled out.

The oxalate was prepared by adding a solution of oxalic acid in absolute ethyl alcohol to a concentrated solution of angiotonin in absolute alcohol. A precipitate formed immediately. Addition of ether to the mother liquors threw down additional precipitate. The oxalate was recrystallized from a mixture of alcohol and water.

## *Animal Assay*

Cats were employed as test animals. Ethyl urethane produces a moderately satisfactory anesthesia, but cats pithed under ether proved the most sensitive and satisfactory

preparation. The animal was quickly anesthetized, the vagi cut, tracheal and carotid cannulae inserted and pithed very slowly through the inner canthus of the eye. Injections were made into the femoral vein. The anticoagulant in the manometer tubing was pontamine-fast pink or heparin. For the first 5 to 10 minutes after pithing and discontinuing administration of ether, the pressor responses are not nearly so great as after this interval had elapsed. The most desirable preparations are those in which the blood pressure is maintained at 30 to 40 mm. Hg.

## RESULTS

*Solubility.*—Angiotonin is soluble in water, alcohol, and propylene glycol. It is insoluble in ether, petroleum ether, and amyl alcohol both in acid and alkaline media.

*Heat Stability.*—Angiotonin is not destroyed by boiling for 1 hour at pH 1, but at pH 10 it is destroyed. Hydrolysis with 10 per cent $H_2SO_4$ for 4 hours destroys it.

*Effect of Oxidizing and Reducing Agents.*—When boiled for 5 minutes with a few drops of superoxol or bromine water, the fluorescence and pressor activity disappear. Treatment with nitrous acid also destroys the fluorescence and activity. Sodium hydrosulfite causes the fluorescence to disappear but the pressor activity is not lost even when reoxidation by air is prevented by a layer of oil. Angiotonin itself has strong reducing properties as it decolorizes potassium permanganate in the cold.

*Precipitation Reactions.*—In aqueous solution, angiotonin is not precipitated by trichloracetic, picric, or flavianic acids, silver nitrate in acid or alkaline media, or mercuric chloride. It is precipitated by phosphotungstic acid.

In alcoholic solution angiotonin is precipitated by oxalic and picric acids. It is also precipitated from alcoholic solution by means of a large excess of ether, especially when the solution is made acid with glacial acetic acid or alkaline with ammonium hydroxide. Angiotonin is adsorbed on Lloyd's reagent in acid media and eluted in alkaline media.

*Fluorescence.*—Angiotonin solutions have a green fluorescence in ultraviolet light in acid or alkaline media. The fluorescence appears to parallel the pressor activity, for treatment with oxidizing agents caused both to disappear. Hydrosulfite abolished the fluorescence, but when the mixture was injected into animals it still retained its pressor action. Presumably the angiotonin was reoxidized in the body.

The ultraviolet absorption spectra showed general light absorption with no definite maxima or minima.

*Color Reactions of Angiotonin.*—Color reactions of angiotonin are shown in Table I. The only test that was definitely positive was the Sakaguchi

test for arginine (guanidine groups). However, the reagent used by Weber (1927) for the determination of guanidine-like substances in blood and urine did not produce a color with angiotonin.

*Pressor Action.*—Angiotonin produces a sharp rise in blood pressure similar to that of adrenaline, but usually slightly more prolonged when injected intravenously in a single dose. It differs from renin in that the rise appears much more quickly, is steeper and less prolonged (Text-figs. 1 and 2). If angiotonin is infused, the pressure may be maintained at an elevated level for at least one-half hour. We have not attempted to keep it elevated longer.

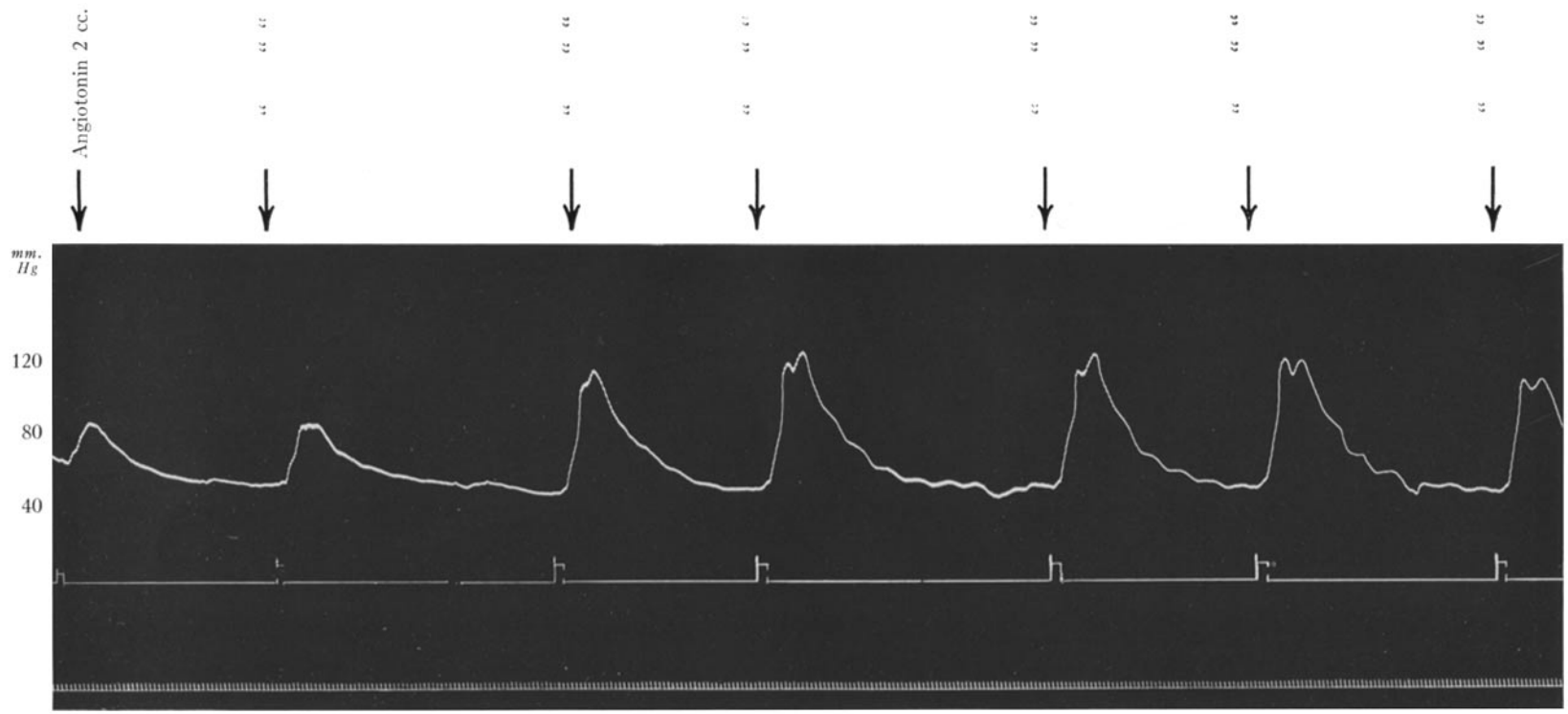
TABLE I

*Color Reactions of Angiotonin*

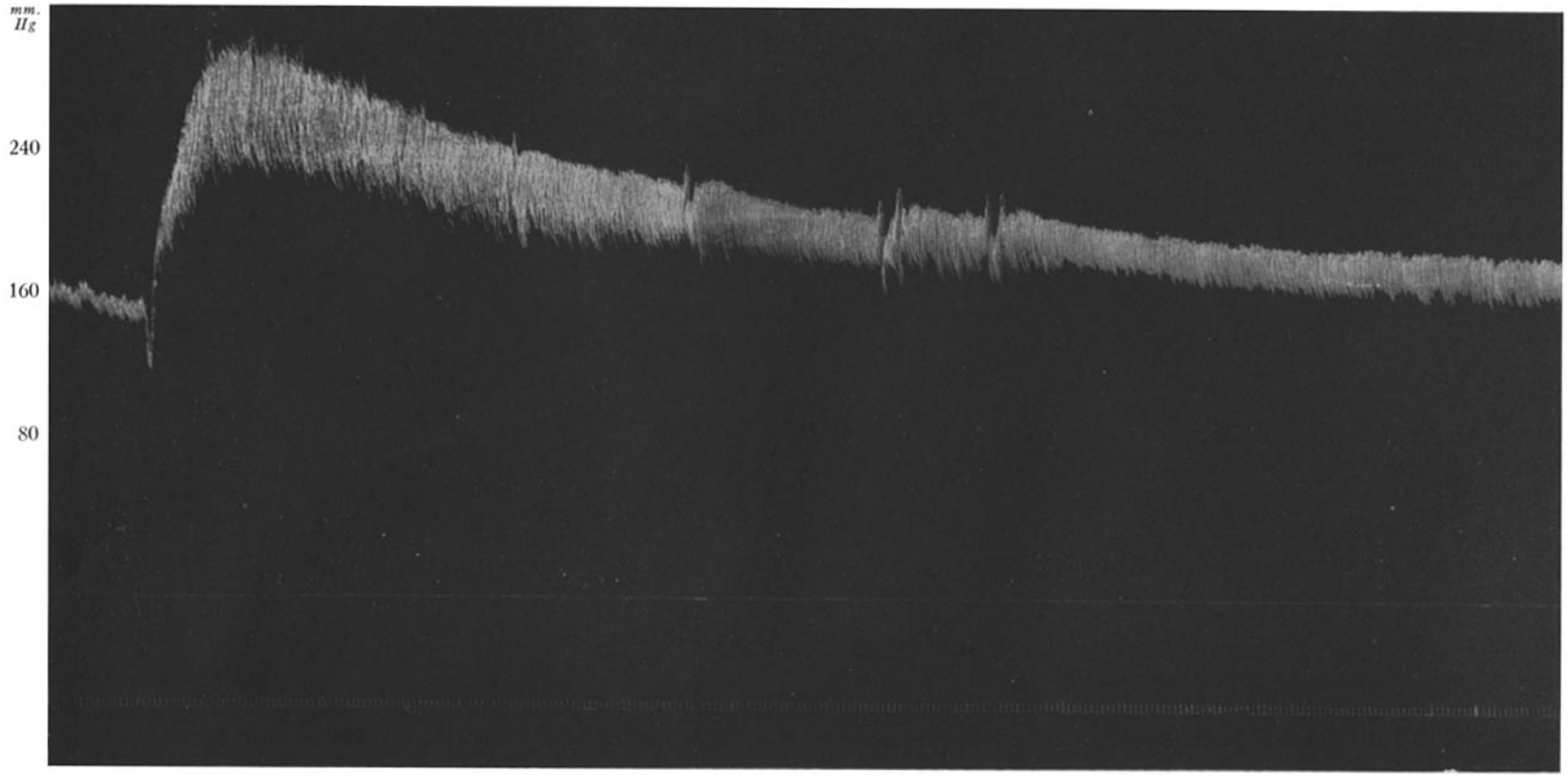
| Reaction | Group | Result |
|---|---|---|
| Biuret | Peptide linkage | — |
| Ninhydrin | Free carboxyl and amino group | — |
| Millon | Tyrosine | — |
| Xanthoproteic | Benzene nucleus | — |
| Ehrlich's benzaldehyde | Tryptophane | — |
| Ehrlich's diazo | Histidine, tyrosine | ? |
| Sakaguchi | Arginine (guanidine group) | ++ |
| Weber reagent | Guanidine-like substances | — |
| Reduced sulfur | Cystine, cysteine | — |
| Molisch | Carbohydrate | ? |
| Bial's orcinol | Pentose | — |
| Dische's indole | " | ? |
| Naphthoresorcinol | Glucuronates | ? |
| Phloroglucinol | " | — |
| Diphenylamine | Levulose, nitrates, nitrites | — |
| Bromocyanogen | Pyridine | ? |
| Vulpian | Adrenine | — |
| Comessatti | " | — |
| Ewins | " | — |

*Effect of Pithing and Anesthesia.*—If ether is employed as anesthetic and then blown off after the animal is pithed, the pressor response to angiotonin is greatly increased, often as much as 4- to 6-fold, as compared with the response before pithing. In such a preparation it is not possible to determine whether the increase in sensitivity is due to loss of anesthetic or to the pithing. It appears probable that it is due to the former because if the animal is pithed under ethyl urethane, there is but slight increase.

*Occurrence of Tachyphylaxis.*—Often repeated single intravenous injections of *renin* into anesthetized cats and dogs invariably cause the pressor



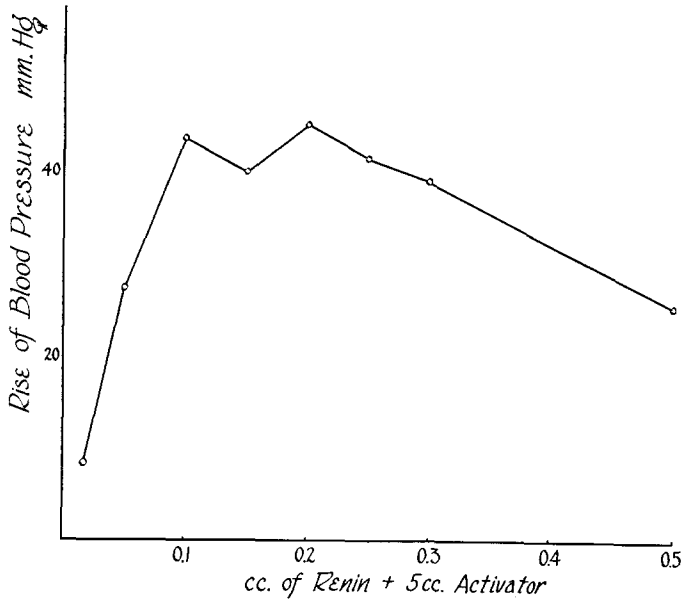

TEXT-FIG. 1. The effect of intravenous injection of angiotonin on the arterial pressure of a pithed cat. Time marker = 6 seconds.




TEXT-FIG. 2. The effect of intravenous injection of renin on a cat under ethyl urethane anesthesia. Time marker = 6 seconds.

response to diminish progressively until even large doses elicit no rise in blood pressure. This phenomenon has been termed tachyphylaxis. Angiotonin, unlike renin, causes only mild tachyphylaxis. As many as 11 injections have been given with but slight lessening of the response. However, some reduction ultimately occurs.

*Action of Drugs on the Pressor Action of Angiotonin.*—It has been repeatedly observed that while there is similarity in the appearance of the curves produced by injecting angiotonin and adrenalin the similarity extends little further. In the occasional animal in which the responses to

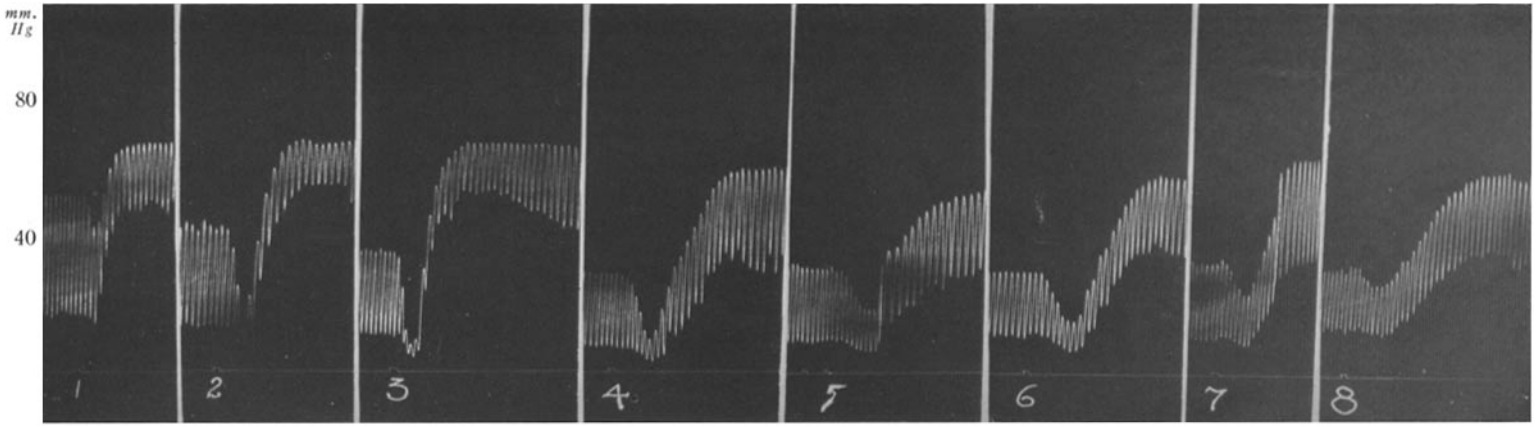



TEXT-FIG. 3. The amount of renin required to produce maximum pressor effect in a pithed cat when mixed with 5 cc. of activator. Renin and activator were incubated together at 40°C. for 10 minutes and then boiled.

even fairly large doses of adrenalin are depressor, angiotonin produced pressor responses.

Cocaine in large doses (6 mg.) greatly enhances the action of adrenalin but not that of angiotonin. Conversely, cocaine abolishes the action of tyramine but not that of angiotonin. For example, 9 injections of angiotonin elevated arterial pressure 20, 20, 18, 18, 14, 14, 10, 10, 10 mm. Hg, and tyramine (1 mg.) 36 mm. Hg. Now, 2 mg. of cocaine was given and tyramine produced only a 6 mm. rise while angiotonin elevated the pressure 14 mm., slightly, but not significantly, more than before the cocaine was administered.

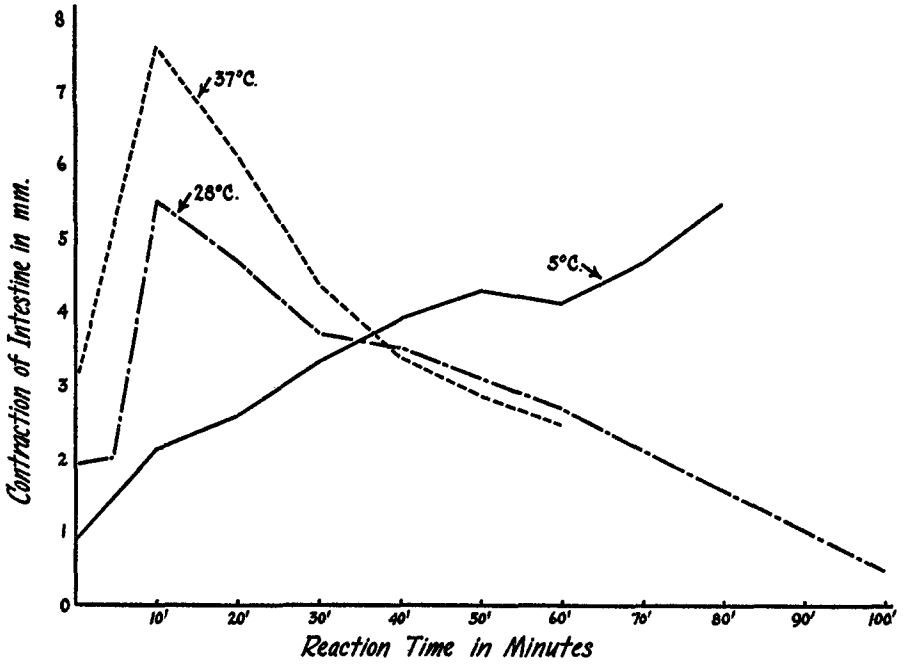
Atropine ($\frac{1}{50}$ grain) was administered intravenously to a pithed cat and




TEXT-FIG. 4. The effect of angiotonin on segment of rabbit's ileum suspended in Tyrode's solution (35 cc. cup). (*1*) Angiotonin 0.5 cc. = 16 mm. rise, (*2*) 1.0 cc. = 24 mm., (*3*) 1.5 cc. = 32 mm., (*4*) 0.5 cc. = 32 mm., (*5*) 0.25 cc. = 27 mm., (*6*) 0.25 cc. = 29 mm., (7) 0.25 cc. = 31 mm., (8) 0.10 cc. = 29 mm.

followed in 1 minute by angiotonin but no difference in response was observed from the control injection. Stilbestrol (20 mg.) also did not alter the response.

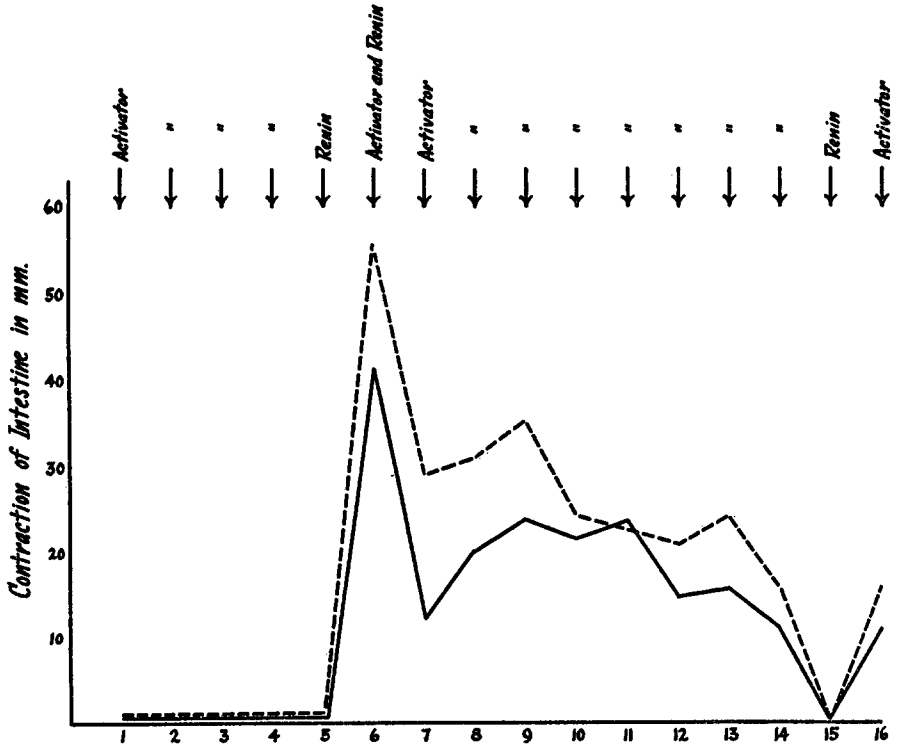
*Suprarenalectomy.*—Removal of both suprarenals in pithed animals did not affect the response to angiotonin injected 5 to 20 minutes after the operation.




TEXT-FIG. 5. Effect of time and temperature on reaction between renin and activator as measured by contraction of rabbit's intestinal segment in Tyrode's solution. Renin and activator were mixed in the proportion of 1 to 9 and 1.0 cc. of the mixture added to 40 cc. of Tyrode's solution in the bath.

*Proportion of Renin and Activator Required to Produce Maximal Amounts of Angiotonin.*—Three experiments were performed to determine in a semiquantitative fashion the amount of renin required to give maximal yields of angiotonin. The proportion found was roughly 3 parts renin to 100 parts of activator. This proportion cannot be taken to represent a stoichiometric relationship in the chemical sense because of the many variables present, but it has proved very useful at this stage of the investigation, especially because of the great saving in the amount of renin employed to prepare angiotonin.

*Effect of Renin on Angiotonin.*—Renin was added to angiotonin in the proportion of 1 to 10 and incubated at 40°C. for varying lengths of time and then boiled 5 minutes. The following is a typical experiment. The rise of blood pressure of a cat was 40 mm. Hg after the control injection of angiotonin. 15 minutes after addition of renin to the angiotonin the same dose elevated arterial pressure 24 mm.; 30 minutes, 16 mm.; 45 minutes,




TEXT-FIG. 6. The effect of activator (1.8 cc.) and renin (0.5 cc.) alone and after having reacted with each other on rabbit's intestinal segment bathed in Tyrode's solution (40 cc.). Ordinates represent the contraction of the intestine in mm. Abscissae represent the successive additions of the substances indicated on the graph.

4 mm.; 60 minutes, 0 mm. Hg. In a second experiment employing another sample of renin and activator the loss of pressor activity was slower: 5 minutes after addition of renin, 48 mm. rise of pressure; 10 minutes, 56 mm.; 20 minutes, 46 mm.; 30 minutes, 32 mm.; 60 minutes, 16 mm. Hg.

*Effect of Angiotonin on Isolated Intestine.*—Angiotonin was added to oxygenated Tyrode's solution bathing segments of rabbit's duodenum and ileum. It caused powerful contraction with little change in the rhythmic move-

ment. The magnitude of the contraction did not vary directly with the amount of angiotonin added. After several additions of angiotonin, the tissue became sensitized to it and responded with large contraction even to very small amounts of angiotonin.

*Effect of Time and Temperature on the Amount of Angiotonin Produced by the Interaction of Renin and Activator.*—Renin and activator were mixed in the proportion of 1 to 9 and incubated at 5°C., 28°C., and 37°C. and the amount of angiotonin ascertained by its contracting action on rabbit's intestine. At 5°C., 80 minutes were required to produce the same action that occurred in 10 minutes at 28° or 37°C.

*Effect of Renin and Activator Alone and Combined on Isolated Intestine.*—Activator alone in amounts of 1.8 cc. added to the bath (40 cc.) containing the intestinal segment produced no contraction. Also renin in doses of 0.5 cc. was ineffective. But if renin and activator in the proportion of 17 to 1 (1.8 cc. total) were allowed to react for 10 minutes at 38°C. and then added, powerful contraction occurred. After thorough washing of the segment, repeated additions of activator now caused marked contraction.

*Effect of Angiotonin on Rabbit's Ear Vessels.*—Perfusion with pulsatile pressure of an isolated rabbit's ear vessels with Ringer-Locke's solution has been shown by Page (1939) to be an excellent method for measuring the pressor action of renin plus activator. Angiotonin in pure form also causes marked constriction.

## DISCUSSION

A highly active, water- and alcohol-soluble, basic pressor substance is formed when renin and renin-activator interact, for which we suggest the name "angiotonin." Since crystalline picrates, and oxalates have been prepared from it, it is probably a base. It is easily oxidized by potassium permanganate and hydrogen peroxide in the cold, and its pressor activity is unaffected by reducing agents such as hydrosulfite. The only strongly positive color reaction which we have found is the Sakaguchi reaction for arginine. Purified renin also gives this reaction strongly according to Helmer and Page (1939). Since the Sakaguchi reaction is positive, it is especially interesting that the Weber reaction for guanidine-like substances is negative. Angiotonin differs sharply from both renin and activator in that it can be boiled without loss of activity. It is acid-stable but alkali-labile. Angiotonin is fluorescent, and fluorescence and pressor activity seem to parallel one another.

From the evidence which we now have, it appears that angiotonin is an intermediate rather than an end product in the interaction of renin and

activator, because incubation of renin with angiotonin causes the destruction of angiotonin. When renin and activator are incubated at 40°C. together, the maximum yield of angiotonin is produced within 30 minutes or less. After this time the amount steadily diminishes.

The pressor action of angiotonin is marked; for example, 0.5 mg. of the picrate elevated the arterial pressure of a pithed cat 88 mm. Hg. The interesting difference exists between the pressor action of angiotonin and renin in that the former produces a sharp, immediate rise, while renin causes a slow one which has a definite latent period before the rise occurs (Text-figs. 1, 2). This difference we believe to be due to the fact that the reaction between renin and activator is relatively slow, hence angiotonin is liberated slowly.

Renin itself is not a pressor substance as was shown by Kohlstaedt, Page, and Helmer (1939), for they found that when purified renin prepared by the method of Helmer and Page (1939) was perfused through a dog's tail or rabbit's ear with Ringer's solution, no constriction occurred. It was only after the addition of renin-activator that the pressor action was manifest. Since renin-activator is normally present in blood, the false impression is given that renin itself is a pressor substance.

We suggest that renin is an enzyme for the following reasons: (*a*) it is a protein, (*b*) it is heat-labile, (*c*) when it reacts with activator the action is slow (Text-fig. 5) and affected by temperature (Text-fig. 5), (*d*) it appears to be required in very small amounts relative to activator (Text-fig. 3). The latter reason can only be tentatively accepted because activator has not been sufficiently purified to be certain of the proportion. However, since the proportion is of the order of 100 of activator to 3 of renin, there is some justification for the statement. If renin is an enzyme, then it seems reasonable to suppose that activator is the substrate on which it acts. It is possible, however, that renin is the substrate, and activator, the enzyme.

Since angiotonin is a product of the interaction of renin and activator and is strongly pressor, there is little reason to doubt that it causes the rise in arterial pressure when renin is injected, and that its maintenance is due to the relatively slow liberation of angiotonin from the reaction between renin and activator.

Angiotonin appears to act primarily on the peripheral blood vessels because pithing does not abolish its action and it produces constriction of the blood vessels of isolated organs. The suprarenal glands do not enhance or decrease its action in acute experiments.

Angiotonin does not resemble any of the usual pressor agents in its re-

sponse to drugs. Unlike renin, many repeated injections are required to lessen the pressor response, so called tachyphylaxis. The mechanism of the development of tachyphylaxis to angiotonin is somewhat different from that of renin. Page (1939) has shown that the reason the pressor response to renin fails so rapidly is exhaustion of activator and development in the animal of an inhibitor. In rabbit's ears perfused with blood, tachyphylaxis is due chiefly to exhaustion of activator. The problem of angiotonin tachyphylaxis will be the subject of a separate communication.

The action of angiotonin in causing strong contraction of the intestine is an interesting one and contrasts sharply with the action of adrenalin. Activator and renin added separately cause no contraction, but when mixed and incubated, the reaction product—angiotonin—causes marked contraction. After the first addition of angiotonin the intestine is altered so that it then responds not only by contraction to each addition of activator, but also to progressively smaller amounts of angiotonin.

The response of the intestine is greatly increased by reducing to one-fourth the concentration of potassium in Tyrode's solution. We have no explanation for this fact.

It is suggested that renin is an enzyme contained in the kidneys without pressor properties, which interacts with renin-activator contained in the blood to form angiotonin, a highly active pressor substance from which several crystalline derivatives have been prepared. The reaction between renin and activator to produce angiotonin may provide the body with a humoral method of regulation of arterial pressure of great precision because of the several components in the system subject to its regulation.

## SUMMARY

1. Renin reacts with renin-activator to form a strong pressor substance which is heat-stable, water- and alcohol-soluble, fluorescent, acid-stable, and alkali-labile. It is a reducing substance and is destroyed by strong oxidizing substances. It forms crystalline salts with oxalic and picric acids. The color reaction for arginine is the only one found to be strongly positive. It is suggested that this substance be called angiotonin.

2. Angiotonin produces a sharp, immediate rise in arterial pressure when injected intravenously. Pithing and dissipation of the anesthetic appear to increase the response. Tachyphylaxis occurs, in contrast to renin, only after many single doses.

3. The responses to adrenaline and angiotonin do not parallel one another.

Cocaine, atropine, and stilbestrol do not affect the pressor action of angiotonin. Suprarenalectomy in brief experiments is also without effect.

4. Maximal amounts of angiotonin result when the proportion between renin and activator is roughly 3 to 100. This is not a stoichiometric relationship in the chemical sense. The temperature suitable for good yields is about 38°C., and the time of reaction from 10 to 20 minutes.

5. Renin destroys angiotonin when incubated with it.

6. Angiotonin causes marked contraction of intestinal segments of rabbits without reducing their rhythmic motion. It sensitizes the intestine to further doses of angiotonin and alters the intestine such that renin-activator contracts it. Angiotonin also constricts the vessels of a rabbit's ear perfused with blood or Ringer's solution.

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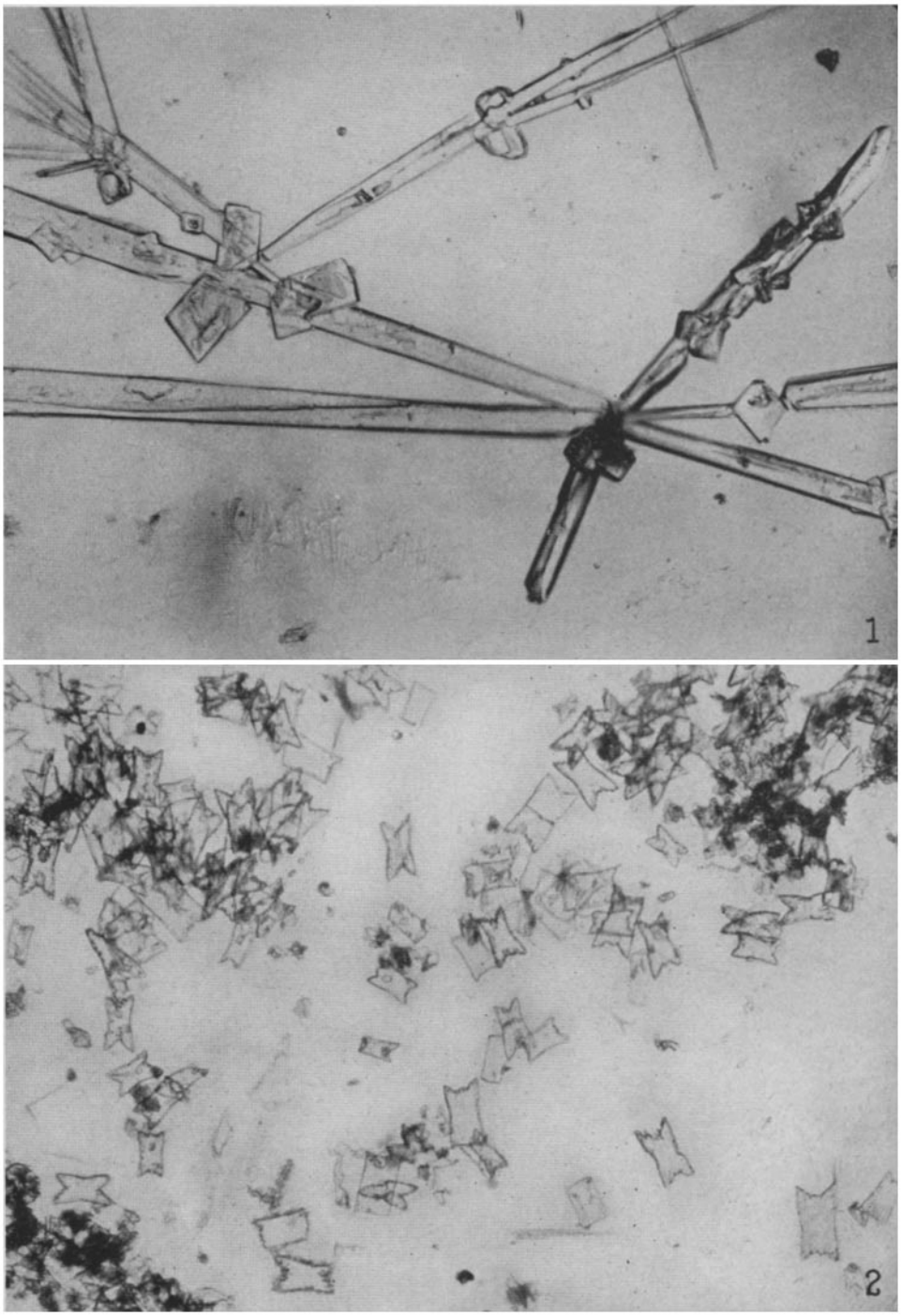
## EXPLANATION OF PLATE 2

FIG. 1. Oxalate of angiotonin crystallized from water.

FIG. 2. Picrate of angiotonin crystallized from alcohol.




(Page and Helmer: Crystalline pressor substance (angiotonin))