and then remains at this level. In aqueous solution under optimum conditions the maximum value attained corresponds to approximately a 50% conversion and then slowly decreases until the absorption approaches that of *allo*-gibberic acid (max. = 266 mμ, ε = 320)[4]. Furthermore, gibberellenic acid yields *allo*-gibberic and gibberic acid when treated under the acidic conditions described[4] for the preparation of these two acids from I.

The above observations strongly indicate that gibberellenic acid is an artifact produced from I by acid catalysis during the fermentation and/or isolation process. Accepting the proposed structure[1] for gibberellic acid (I), gibberellenic acid may be formulated as IV, its formation from I presumably proceeding by way of the carbonium ion intermediate (V)[5]. No strictly analogous model for comparison of ultraviolet absorption appears to be available but the observed absorption of IV is not incompatible with the values reported for diene systems[6]. The resistance of the tetra-substituted double bond in IV towards hydrogenation (see above) is not surprising and finds its parallel in the observed resistance to catalytic reduction of an analogous double bond in $\Delta^{8,9}$-bicyclo(4·3·0)nonene-5,6-dicarboxylic[7]. Although the evidence presented here does not rule out the alternate structure (VI) for gibberellenic acid, the ready conversion of this acid by decarboxylation and dehydration to the aromatic *allo*-gibberic acid (II) supports structure IV.

Although the data reported here support the contention that gibberellenic acid (IV) is derived from I by acid catalysis, the conversion of I to IV in aqueous acid on a preparative scale was not feasible because of the low solubility of I and the formation of further degradation products under these conditions. On the other hand, acid methanolysis followed by neutralization with methanolic sodium methoxide provided a new crystalline dibasic acid (m.p. 188–190°, gas evolution) which likewise has an absorption maximum at 253 mμ (ε = 19,000). This acid was recovered unchanged after a 12 h treatment with 1 *N* sodium hydroxide. This observation together with the analytical data (Calculated for $C_{20}H_{24}O_6$: C, 66·65; H, 6·71; O–CH_3(1), 8·61; mol. weight, 360. Found: C, 66·11; H, 7·25; O–CH_3, 7·89; mol. weight by electrometric titration, 360) allow the formulation of this acid as the methyl ether of gibberellenic acid (VII). The alternate structure (VIII) can be ruled out since its formation requires an unlikely carbonium ion intermediate. That this methyl ether-acid is correctly represented by VII was demonstrated by its conversion to *allo*-gibberic acid (II), employing the mild acidic conditions[4] which effect the conversion of I to II. Vigorous acid treatment[4] of VII yields gibberic acid (III). The direct conversion of IV to VII using methanolic sulfuric acid could not be realized indicating that the conversion of I to VII does not involve IV as an intermediate.

Gibberellin A_1 has been reported[8] to be a dihydrogibberellic acid having one double bond and should therefore be IX or X. As a sample of gibberellin A_1[9] in acidic solution did not develop the typical absorption at 253 mμ, it must possess structure IX. This same structure has recently been proposed by SUMIKI *et al.*[8] on the basis of ozonolysis experiments.

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The Lilly Research Laboratories, Indianapolis, Indiana, July 30, 1957.

Zusammenfassung

Unter den Fermentationsprodukten von *Fusarium moniliforme* findet sich neben der bekannten Gibberellinsäure als Nebenprodukt die Gibberellensäure. Identifizierung und Charakteristika dieser Säure (IV) werden beschrieben. Für Gibberellin A_1 wird die Struktur (IX) angegeben.

[4] B. E. CROSS, J. chem. Soc. *1954*, 4670.

[5] This transformation closely resembles the acid catalyzed conversion of ψ-santonin to ψ-santonic acid. – N. M. CHOPRA, W. COCKER, J. T. EDWARD, T. B. H. MCMURRY, and E. R. STUART, J. chem. Soc. *1956*, 1828 and other references listed.

[6] L. L. FIESER and M. FIESER, *Natural products related to Phenanthrene* (Reinhold Publishing Corporation, New York 1949), p. 185.

[7] A. T. BLOMQUIST, J. WOLINSKY, Y. C. MEINWALD, and D. F. LONGONE, J. Amer. chem. Soc. *78*, 6058 (1956). – We thank Dr. BLOMQUIST for the generous gift of this model compound.

[8] Y. SETA, H. KITAMURA, N. TAKAHASHI, and Y. SUMIKI, Bull. agric. chem. Soc. Japan *21*, 73 (1957). This structure has recently been confirmed by Dr. MAC MILLAN and his group. (Personal communication to K.G.)

[9] We thank Dr. F. H. STODOLA of Peoria, Illinois, for the generous gift of a sample of pure gibberellin A_1.

Release of Bradykinin as Related to the Esterase Activity of Trypsin and of the Venom of *Bothrops jararaca*

In a previous paper[1] we have shown that the bradykinin releasing activity of the venom of *Bothrops jararaca* is not related to its proteolytic activity on casein. Two apparently distinct proteolytic activities were demonstrated in the venom depending upon their different heat tolerance. While the activity on casein was found to be easily destroyed on a few minutes heating in boiling water, the remaining esterase activity on benzoyl-L-arginine methyl ester (BAME) proved to be more thermostable and only partially destroyed[2]. As shown by SCHWERT *et al.*[3] trypsin besides its proteolytic activity on casein also displays esterase activity on BAME, the latter being correlated to its amidase activity on benzoyl-L-arginine amide[4]. The venom of *Bothrops jararaca* has also been shown to act on casein and the benzoyl-L-arginine ethyl ester[5]. It therefore seems possible that the common mediator for bradykinin release of both trypsin and the snake venom may be their respective esterase activities. In the present work we have shown that such a correlation can be demonstrated. If trypsin inhibitor is removed by applying the denatured plasma as a substrate a direct relationship is found between the bradykinin releasing effect of trypsin and the heated venom of *Bothrops jararaca* when compared with their respective esterase activities on BAME.

Heating a solution of the venom in a boiling water bath for 1–3 min[1] was enough to destroy completely its

[1] U. HAMBERG and M. ROCHA E SILVA, Arch. int. Pharmacodyn. *110*, 2 (1957).

[2] U. HAMBERG and M. ROCHA E SILVA, Arch. int. Pharmacodyn. *110*, 2 (1957); Proc. 20th Int. physiol. Congr. Brussels *390* (1956).

[3] G. H. SCHWERT, H. NEURATH, S. KAUFMAN, and J. E. SNOKE, J. biol. Chem. *172*, 221 (1948).

[4] M. BERGMANN, J. S. FRUTON, and H. POLLOK, J. biol. Chem. *127*, 643 (1939).

[5] C. R. DINIZ and H. F. DEUTSCH, J. biol. Chem. *216*, 17 (1955).

proteolytic activity on casein and reduced to about 50% its esterase activity on BAME. However if heating was continued a slow but continuous drop in the residual esterase activity was observed. Complete destruction was obtained after a prolonged heating for 90 min in a boiling waterbath. This would indicate (Fig. 1) that we are dealing with a complex enzymatic mixture and that at least three different activities are involved: (1) A thermolabile activity as measured on casein; (2) a thermolabile esterase activity, possibly connected with activity (1), and (3) a more thermostable esterase component, concerned with the release of bradykinin.

The capacity of the venom of releasing bradykinin when incubated with fresh plasma, first increased after a short heating of the venom, concomitantly with a drop in proteolytic activity, until a maximum is attained after 1–3 min of heating the venom. Thereafter the yield decreased in parallel with the esterase activity. The initial increase in the yield of bradykinin might be explained by the fact that the bradykinin destroying activity of the venom disappears with its proteolytic activity on casein.

The clotting power of the venom decreased sharply when heat was applied. There was a marked initial drop after 1 min heating, to about 30% of the initial value, followed by a slow decrease in parallel with the relatively thermostable esterase activity (Fig. 1). It can therefore be concluded that this residual esterase activity correlated to bradykinin release also is effective in bringing about the coagulation of the blood.

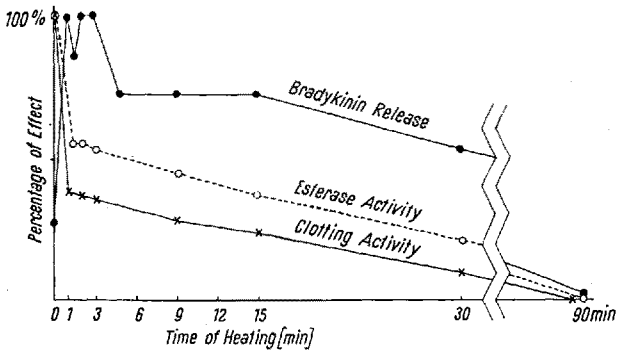

Fig. 1.—Effect of heat denaturation upon the bradykinin releasing and clotting activities of the venom of *Bothrops jararaca*, as related to its esterase activity on BAME. In abscissae, time in minutes of heating a 0·5% saline solution of the venom at pH 6 in a boiling waterbath. Temperature at the end of heating periods 82–95°C. The proteolytic activity on casein as measured by absorption in U.V. decreased to zero after 1 min heating (the curve is not presented in this graph).

After 3 min heating of the venom residual esterase activity was found to be about $^1/_{20}$ of that of trypsin. Since destruction of bradykinin by the venom was prevented by the heating and since the bradykinin destroying effect of plasma, as well as the trypsin inhibitor also were removed by heat denaturation of the plasma[2], a comparison of the esterase activity with the bradykinin releasing effect of the venom was made possible. The experiments were performed by applying equipotent esterase activities (BAME) of trypsin and heated venom on denatured plasma. Equal amounts of bradykinin were found to be released with both agents showing a parallelism between the relatively thermostable esterase activity of the venom and its bradykinin releasing effect. A striking difference was found regarding the specificity of the proteolytic activity of both agents. Although the concentration of split products with absorption in U.V. light increases with time and in parallel with bradykinin release when trypsin is used, no measurable amounts of protein split products are obtained with the heated venom. In another series of experiments, increasing concentrations of venom and trypsin were incubated with denatured plasma, and the ratios of their respective esterase activity (BAME) were determined (Fig. 2). In each case equal bradykinin release was obtained with both agents.

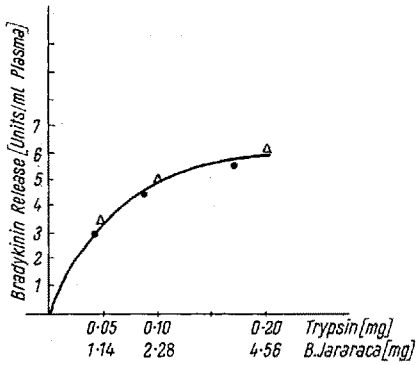

Fig. 2.—Bradykinin released from denatured plasma proteins with increasing equipotent (BAME) esterase concentrations of trypsin and the heated venom (mg/ml plasma).

These findings agree with those described by HOLTZ and RAUDONAT[6] who were able to separate by fractionation with ammonium sulphate a thrombin-like fraction (Koagulin) from a proteolytic fraction which activates prothrombin. The bradykinin releasing activity was present in the non-proteolytic 'Koagulin' fraction of the *Bothrops* venom.

In conclusion: Fractionation by heat of the venom of *Bothrops jararaca* gives a fraction with the specific activity on benzoyl-L-arginine methyl ester (BAME) which can be directly correlated to bradykinin release from fresh or denatured plasma and with the clotting power on fresh plasma. When compared with the effect of trypsin equal ratios are obtained with the heated venom for the bradykinin releasing effect and the esterase activity on BAME. The high specificity of the venom in releasing bradykinin would indicate that bradykinin is held in a terminal position in the precursor molecule.

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Zusammenfassung

Die proteolytische Aktivität des Giftes von *Bothrops jararaca* wird durch Erhitzen leicht zerstört und dabei eine Esterase mit grösserer Hitzeresistenz abgetrennt. Nach Hitzefraktionierung des Giftes steht die Aktivität dieser Esterase in direktem Verhältnis zum abgegebenen Bradykinin und zur Gerinnung des frischen Plasmas.

[6] P. HOLTZ and H. W. RAUDONAT, Arch. exp. Path. Pharmak. 229, 113 (1956).

[7] Fellow of the C.A.P.E.S., Rio de Janeiro, Brazil.