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Formation of a Pharmacologically Active Substance from Plasma Protein.* (24537)

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A number of investigators(1,2) have shown that vasodilator polypeptide, bradykinin, can be produced by incubating plasma protein with crystalline trypsin. Also that an enzyme capable of producing bradykinin upon incubation with plasma protein can be released from salivary gland cells by stimulation of the chorda tympani(3) and that bradykinin so produced has a role in regulation of blood flow in the submandibular salivary gland. It occurred to us that the enzyme α-amylase might be important in production of bradykinin, since salivary glands are rich in α-amylase. Furthermore, fraction IV-4 of plasma protein which contains bradykininogen, the precursor of bradykinin, is rich in glycoprotein which could well be susceptible to hydrolysis by α-amylase. Therefore it seemed conceivable that the action of α-amylase on glycoprotein might lead to production or activation of bradykinin. Saliva incubated with blood produced vasodilator principle bradykinin(3) and α-amylase is present in saliva along with small amounts of other enzymes.

Materials and methods. Fraction IV-4 of plasma protein was obtained through joint cooperation of American Red Cross and E. R. Squibb. Enzymes and enzyme inhibitors were obtained from commercial sources except salivary amylase, which was prepared from pooled saliva and partially purified by the method of Bernfeld(4). Detection and extent of formation of the pharmacologically active material in the incubation mixture was measured by ability to contract isolated segments of guinea-pig ileum suspended in 10 ml organ bath and attached to frontal writing lever for recording on smoked drum of kymograph. The bath fluid was Tyrodes' solution at 37°C. The effect of pharmacologically active polypeptides on blood pressure was studied in dogs and rabbits anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg) and urethane (1-1.5 g/kg), respectively. Pressure was recorded from cannulated carotid artery with a mercury manometer. All injections were made *via* cannulated femoral vein. Formation of pharmacologically active material was carried out by incubating equal volumes of 0.5% α-amylase and 0.5% fraction IV-4, both dissolved in Tyrodes solution at 37°C. At appropriate times aliquots were removed and tested for activity on the guinea pig ileum. The active material was in contact with the tissue for 60 seconds. Aliquots of the incubation mixture, removed at appropriate times, were also injected I.V. into anesthetized dogs and rabbits for measurement of effects on blood pressure. Formation of

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bradykinin was accomplished by incubating equal volumes of 0.5% fraction IV-4 and 0.03% crystalline trypsin both dissolved in Tyrodes' solution at 37°C. When enzyme inhibitors were used they were preincubated with appropriate enzymes for 15 minutes before mixing with the plasma protein fraction. Enzyme inhibitors were used in concentration equal to that of the enzyme.

Results. A pharmacologically active substance was formed by incubating α-amylase (NBC #9342) with fraction IV-4 of plasma protein. Maximum production of the active material was attained after approximately 20 minutes' incubation. This is contrasted with formation of bradykinin by action of crystalline trypsin on fraction IV-4 of plasma protein in which time of maximum formation was between 2 and 5 minutes incubation. Rate of destruction of the active substance produced by incubating α-amylase with fraction IV-4 of plasma protein, by enzymes present in the incubation mixture, proceeded much more slowly than its formation, requiring approximately 100 minutes for the activity to become negligible. This was contrasted with rate of destruction of bradykinin in crystalline trypsin:fraction IV-4 incubation mixture in which it was found that bradykinin activity was negligible after 20 minutes incubation. Prolonged incubation of enzymes or substrate alone did not produce any active product. All Cohn fractions of plasma protein except fraction IV-4 were inactive as substrates for enzymatic formation of the pharmacologically active substance.

If a specific α-amylase inhibitor‡ was added to the fraction IV-4:α-amylase incubation tube, in concentration equal to that of the α-amylase present, formation of the active substance was completely inhibited. Also, trypsin inhibitor‡ prevented formation of bradykinin when added in concentration equal to that of the crystalline trypsin. In addition, α-amylase inhibitor had no effect on formation of bradykinin and trypsin inhibitor had no effect on formation of the active substance obtained by incubating α-amylase with fraction IV-4 of plasma protein. It would therefore seem that the pharmacologically active substance was different from bradykinin. This possibility was confirmed by experiments testing the activity of these substances on dog and rabbit blood pressure. Incubation mixtures of fraction IV-4 and crystalline trypsin when injected into dogs and rabbits, gave the hypotensive response typical of bradykinin. When incubation mixtures of fraction IV-4 and α-amylase were injected into dogs and rabbits a pressor response was obtained. Since this active substance differs from bradykinin we have provisionally named it Substance A.

‡ Obtained from Worthington Biochemicals Corp., Freehold, N. J. The trypsin inhibitor was a 5-times crystallized soy bean trypsin inhibitor.

TABLE I. Comparative Ability of Different Alpha Amylase Preparations to Form a Pharmacologically Active Material when Incubated with Fraction IV-4.

Source of alpha amylase	Time of max formation (min.)	Relative activity*
Nutritional Biochemicals Corp.		
#9342	18	100
#4416	10	43
#4261	6	169
Southeastern Biochemicals	12	122
Mann #A-5074	25	93
K and K Labs. #9307F	5	67
Salivary (partially purified)	10	60

* Calculated using Nutritional Biochemicals #9342 as standard alpha amylase.

Table I illustrates ability of different α-amylase preparations from various sources to form a pharmacologically active material when incubated with fraction IV-4. All of the α-amylase preparations tested were able to produce an active substance. β-amylase was found to be totally inactive under the conditions of our test.

Summary. Evidence has been presented on formation of a pharmacologically active substance from fraction IV-4 of plasma protein by the action of an α-amylase preparation. Formation of the active substance stimulates the isolated guinea pig ileum and produces a pressor response on dog and rabbit blood pressure. It differs from bradykinin since that substance is a vasodilator. The active substance produced by action of α-amylase on fraction IV-4 of plasma protein has been pro-

visionally designated as Substance A. Further studies concerning enzymatic formation as well as purification and characterization of Substance A will be published.

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Serial Cell-Free Passage of a Radiation-Activated Mouse Leukemia Agent.* (24538)

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Mice of the C3H inbred line do not usually develop "spontaneous" leukemia. Although the incidence varies in different laboratories, in our colony of C3H or C3H(f) mice (both of Bittner substrain) the incidence during past 10 years has not exceeded 0.5%(1). However, fractionated total body irradiation (150 r, 4 to 5 times, at weekly intervals) resulted in development of leukemia in over 50% of C3H mice after a latency period of approximately 6 to 7 months. Leukemia thus induced could then be transmitted, by filtrates, into newborn C3H mice; incidence was significant (11%), but not too high, and many extracts were inactive on inoculation tests (2). In our previous studies, some filtrates prepared from spontaneous Ak or C58 leukemias were also inactive on inoculation tests; however, selecting a potent filtrate and passing it serially through newborn mice, resulted eventually in development of a highly potent "passage A" leukemic virus inducing up to 90% leukemia after a latency of 3 to 4 months following inoculation into newborn or suckling C3H mice(3,1). Faced now with an apparently similar situation, we selected one of the more potent extracts among those prepared from radiation-induced C3H leukemias, and passed it serially through newborn C3H mice.

Materials and methods. All mice used were C3H or foster nursed C3H(f) mice, both of the Bittner subline. *Origin of leukemic virus strain.* The donor serving for preparation of the initial filtrate was a C3H female which, at age of 1½ months received a series of total body x-ray irradiation, 150 r each at weekly intervals, for 4 consecutive weeks. Five months after last irradiation, this mouse developed a very large thymic lymphosarcoma, and was then used as donor for preparation of the initial filtrate. *Preparation of filtrates for inoculation.* The leukemic donors were sacrificed by ether inhalation, and, without delay, parts of thymic and mesenteric tumors, peripheral lymph nodes, livers and spleens, were removed aseptically, weighed, and ground by hand in mortar with chilled, sterile physiological saline added, to obtain cell suspensions of 20% concentration. After centrifugation at 3,000 rpm (1,400 x g) for 15 minutes, the supernate was removed and again centrifuged at 9,500 rpm (7,000 x g) for 5 minutes. The second supernate (10 to 12 ml) was mixed with 0.5 ml of 1:2000 dilution of fresh broth culture of *E. coli*, and passed through Selas, porosity 02, porcelain filter candles, under vacuum pressure of approximately 20 mm mercury. All resulting filtrates were bacteriologically sterile, as evidenced by inoculating tryptose phosphate broth incubated for 24 hours; it was reasonable to assume, therefore, that no cells passed through filter candles. Kept at 0°C, most extracts

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