Pharmacological Observations on a Chymotryptic Inhibitor from Potatoes.* (29034)

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The isolation of a powerful chymotryptic inhibitor from potatoes has been reported by Ryan and Balls(1). In anticipation of possible clinical use of this inhibitor studies were made to determine: (I) its effect on chymotrypsin attached to an insoluble animal protein; (II) its acute toxicity, and (III) its effect on immunological responses to chymotrypsin.

I. Bovine blood fibrin was disintegrated in water to form a 1% suspension. Three 1.0 ml portions of this suspension (*i.e., ca* 100 mg dry fibrin), here called A, B, and C were buffered by the addition of 0.10 ml of 0.1 M phosphate, pH 6.0, respectively. Two portions, A and B, each received 0.25 mg of α-chymotrypsin in 0.20 ml water; the third, C, 0.20 ml water only (as a control). After 20 min at 25°C each system was placed in a small filter tube equipped with a fritted glass disc. The liquid was drawn off by suction and the moist fibrin was washed thrice with 2 ml portions of 1% sodium chloride solution. In the case of A, however, the salt solution also contained inhibitor (0.0125%). Thereafter, A, B, and C were all washed twice more with 2 ml portions of plain salt solution, thus removing adhering free inhibitor from A. Each of the 3 cakes of washed fibrin was then suspended in 3.0 ml of 0.1 M phosphate, pH 6.0, for 1.5 hours at 25°C. Thereafter, each was sucked dry on its respective fritted glass disc, and washed 5 times with 2 ml portions of 1% NaCl solution. The exact volume and optical density (at 280 mμ) of each filtrate were measured, and the protein corresponding thereto in the total filtrate of each sample was calculated.

Thus the filtrate from A is a measure of proteolysis in fibrin that had been exposed to chymotrypsin and then washed with dilute inhibitor solution and salt solution. It contained digestion products equivalent to 87 μg of protein. The filtrate from B shows the action of a like quantity of chymotrypsin retained in fibrin that had been washed with salt solution only. It measured 820 μg. The filtrate of C represents material extracted from fibrin by the buffer and salt solution used for washing, without treatment by either enzyme or inhibitor. This filtrate measured 75 μg, almost as much as that obtained in A. It may be concluded, therefore, that exposure to a solution of inhibitor stops the action of enzyme already attached to fibrin and normally active thereon.

II. The use of chymotrypsin in cataract surgery has been reviewed by Drance(2). Approximately 1 ml of a 1:5,000 or 1:10,000 dilution of chymotrypsin is placed in the anterior chamber of the eye, and washed out about 2 minutes later with physiological saline. The presence of a nontoxic inhibitor of proteolysis in the physiological saline would be desirable. In the following toxicity tests the dosages of the chymotryptic inhibitor were based upon the following considerations. The molecular weights of pure chymotrypsin and the inhibitor are approximately 25,000 and 32,000, respectively. The amount of inhibitor required to stop proteolysis is about ⅓ the weight of pure chymotrypsin(3), or 66 μg of inhibitor to inactivate the 200 μg of chymotrypsin in 1 ml of a 1:5,000 dilution of the enzyme. The dosages of inhibitor used in the following toxicity tests are therefore greatly in excess of the amounts of free inhibitor remaining under practical conditions of use for inactivating proteolytic activity of chymotrypsin.

Thirty male Swiss-Webster mice ranging in weight from 22 to 26 g, and 6 albino rabbits were used in acute toxicity tests. In

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every instance the dose of inhibitor administered was dissolved in 0.5 ml of physiological saline. Three mice were injected intravenously with 75 μg, two with 150 μg, 5 with 300 μg, and 5 with 1000 μg of inhibitor.

Three groups of 5 mice each were injected intraperitoneally with doses of 150 μg, 300 μg, and 1000 μg of inhibitor. Three of 5 mice given 150 μg intraperitoneally were sacrificed 24 hours later and examined for evidence of irritation. No peritoneal fluid or evidence of hyperemia was found. The remaining 27 mice given either intravenous or intraperitoneal injections of the inhibitor appeared normal throughout 2 weeks of observation and showed no abnormalities at autopsy.

Since a dilute solution of inhibitor may be potentially useful for inhibiting the proteolytic action of a 1:5,000 dilution of chymotrypsin placed in the anterior chamber of an eye, the following test was made on rabbit eyes. Seventy-five μg of inhibitor were placed in the conjunctival sac of one rabbit eye every day for 5 days. The same experiment was performed on 3 rabbits with 150 μg to give a total of 750 μg, and on 2 rabbits with 300 μg for a total of 1500 μg per rabbit. At no time during the instillations nor one week following the last instillation was there any evidence of irritation compared with the contralateral eyes.

III. Unpublished immunological data obtained by Jansen and Booth‡ of this laboratory in 1950 demonstrated that diisopropyl fluorophosphate (DFP), which inhibits the proteolytic activity of chymotrypsin(4), does not eliminate its antigenic properties. The procedure used by them was as follows: Nine ml of water and 1 ml of a 10% solution of potassium-aluminium sulfate-24 aq (pH 3.8) were added to 108 mg of DFP-inactivated chymotrypsin. The pH was adjusted to 7.0 adding 0.28 ml of 2 N sodium hydroxide followed by an adequate amount of 0.1 N sodium hydroxide. The resulting turbid suspension was stored overnight in a refrigerator. The following day a guinea pig was injected subcutaneously with the equivalent of 10 mg of protein in the above preparation. About 25 days later the intravenous injection of 1 mg of chymotrypsin produced fatal anaphylactic shock in the sensitized guinea pig. It was, therefore, of interest to determine whether the chymotrypsin inhibitor from potatoes would eliminate the antigenicity of chymotrypsin.

Attempts to sensitize female guinea pigs by intraperitoneal injection of chymotrypsin either resulted in death of the animals or failed to give adequate sensitization. Two female guinea pigs injected subcutaneously and 2 injected intramuscularly with chymotrypsin and Freund's adjuvant survived. The following experiment describes results obtained on the only animal showing evidence of sensitization. The Schultz-Dale technique was used to record uterine responses, each uterine horn being suspended in 50 ml of Tyrode solution. After demonstrating that each uterine horn contracted normally upon addition of 0.01 mg of histamine to the Tyrode bath and relaxed upon addition of 0.1 mg of epinephrine, the tissues were washed and the baths filled with fresh Tyrode solution. One uterine horn was challenged by adding 10 μg of chymotrypsin to the Tyrode bath. Contraction typical of antigen-antibody reaction occurred, and relaxation took place upon addition of epinephrine. After washing, the uterine horn no longer contracted when chymotrypsin was added to the bath, but responses to histamine and epinephrine were normal. The other uterine horn was challenged by adding 14 μg of the chymotrypsin-inhibitor complex to the bath. Contraction occurred as when chymotrypsin alone was added to the first uterine horn. The tissue relaxed spontaneously, reacted normally to histamine and epinephrine, but gave no response to subsequent additions of the complex or chymotrypsin to the Tyrode bath. These results suggested that the inhibitor prepared from potatoes inactivates the portion or portions of the chymotrypsin molecule responsible for proteolysis but does not interfere with antigenicity.

To confirm these results and extend observations, 7 female guinea pigs were sensitized

‡ Personal communication from E. F. Jansen.

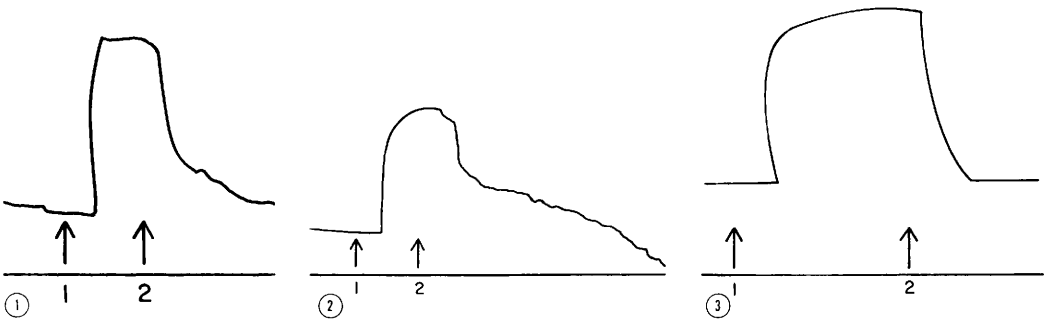

FIG. 1. Uterine reaction of guinea pig sensitized to chymotrypsin. 1. 10 γ chymotrypsin added to excised organ bath. 2. 0.1 ml epinephrine (1:1000) added to excised organ bath.

FIG. 2. Uterine reaction of guinea pig sensitized to chymotrypsin. 1. 14 γ chymotrypsin-inhibitor complex added to excised organ bath. 2. 0.1 ml epinephrine (1:1000) added to excised organ bath.

FIG. 3. Uterine reaction of guinea pig sensitized to chymotrypsin. 1. 10 γ chymotrypsinogen added to excised organ bath. 2. 0.1 ml epinephrine (1:1000) added to excised organ bath.

with single subcutaneous injections of DFP-inactivated chymotrypsin as described above. The results were confirmatory, and even more striking because of the greater sensitivity of the guinea pigs. Typical responses to challenging with chymotrypsin and chymotrypsin-inhibitor complex are shown in Fig. 1 and 2, respectively. Fig. 3 shows the response of a sensitized uterine horn to chymotrypsinogen.

In 2 instances after demonstrating sensitization by challenging one uterine horn with chymotrypsin, 360 μg of inhibitor was added to the Tyrode bath containing the other uterine horn. Five minutes later the tissue was washed and the bath filled with fresh Tyrode solution. When challenged with 10 μg of chymotrypsin the uterine horn contracted, indicating that if the inhibitor had formed a complex with chymotrypsin antibodies the antigen-antibody reaction was not involved.

Following removal of hair from the abdomen of a sensitized guinea pig, 1 ml of a 0.05% solution of Evans blue was injected intravenously. In widely separated abdominal areas intracutaneous injections were made of chymotrypsin, inhibitor, chymotrypsin-inhibitor complex, and chymotrypsinogen, corresponding to 5, 18, 7, and 5 μg, respectively. Blueing failed to occur in any of the injected areas. Since the animal was sensitized, as shown subsequently by contraction of the excised uteri when challenged with chymotrypsin, the experiment was repeated using twice the intravenous dose of Evans blue dye. The intracutaneously injected areas given the complex and chymotrypsinogen showed a one-plus blueing in about 10 min, and all areas except that injected with the inhibitor alone were rated as 2-plus at 1 and 2 hours.

All the results obtained, together with the observation that scratch tests made with the chymotrypsin-inhibitor complex on 2 human volunteers known to be sensitive to chymotrypsin gave positive reactions, show that the inhibitor derived from potatoes does not inhibit antigen-antibody reactions.

Summary. An inhibitor of chymotrypsin isolated from potatoes was found to stop the proteolytic activity of the enzyme on fibrin to which the enzyme had already been firmly attached. The inhibitor was nontoxic to mice when injected intravenously or intraperitoneally in amounts as high as 40 mg per kg. It was also nonirritating when applied repeatedly to the conjunctival sac of rabbits' eyes. However, the inhibitor does not change the antigenicity of chymotrypsin.

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Autoradiographic Localization of Iodide^{125} in the Thyroid Epithelial Cell. (29035)

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Autoradiography has been employed to investigate the localization of inorganic iodide^{131} within the thyroid follicle in several species(1,2). These studies were performed in animals in which the incorporation of iodide^{131} into thyroidal protein-bound iodine^{131} (PBI^{131}) was inhibited by administration of a goitrogen and at a time when thyroidal iodide^{131} was almost equilibrated with serum iodide^{131}. Radioiodide was localized primarily in the follicular lumen and cellular radioiodide concentration was greater than that in blood vessels. It appeared possible that blood vessel and cellular radioiodide concentrations may not have been appreciably different *in vivo*, and that the excess cellular radioiodide may have arisen by diffusion of luminal radioiodide after excision of the thyroid gland. Therefore it seemed possible that the iodide concentrating mechanism might be localized at the apical end of the epithelial cell. Recent studies by Tong *et al*(3), however, have demonstrated that isolated thyroid epithelial cells are able to concentrate radioiodide. We have made systematic investigations of the autoradiographic localization of iodide^{125} in the intact thyroid follicle *in vivo* and here report conditions in which radioiodide is more concentrated in the follicular epithelium than in the follicular lumen.

Methods. Male C_3H mice, 3 to 5 months of age and fed Purina Laboratory Chow since weaning, were used in these studies. Where indicated animals were given 2 mg of propylthiouracil in 0.04 N NaOH by subcutaneous injection 30 minutes before iodide^{125} in order to inhibit the formation of thyroidal PBI^{125}. Under pentobarbital anesthesia mice were given 100-200 μc of iodide^{125} by intraperitoneal injection. Five minutes later glands were quickly removed while still attached to the trachea and plunged into isopentane cooled to about —160°C by liquid nitrogen. Thyroids were dried at —45°C, paraffin embedded in vacuum and sectioned at 6 μ. Sections were mounted permanently in contact with the emulsion on glass slides covered with AR-10 autoradiographic stripping film. After suitable exposure, the "integrated autoradiographs" were developed, fixed and stained with Nuclear Fast Red by methods to be described in detail elsewhere. Control studies with non-radioactive thyroids showed that histologic sections did not cause chemical induction of a latent image and did not significantly modify a uniform latent image produced by soft X-rays.

Results. Organic binding of radioiodide blocked. In about 60% of the 20 thyroids examined at least a few follicles in each gland had autoradiographic images primarily over the epithelial cells 5 minutes after iodide^{125} administration (Fig. 1, a and b). Approximately 20% of the thyroids contained autoradiographic rings associated with a third or more of the thyroid follicles. In the remaining follicles the autoradiographic grain density over the lumen was at least equal to the grain density over the epithelium. There was a much lower density of grains over blood vessels and parathyroid glands. At later times after radioiodide injection our findings were similar to those previously reported by others(1,2).