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29 January 1958

Effect of Trypsin Inhibitor on Passage of Insulin Across the Intestinal Barrier

The finding of trypsin inhibitor in colostrum led to the hypothesis that the physiological role of the inhibitor is to protect the antibodies of colostrum from being digested and thus to facilitate their absorption (*1*). Some circumstantial evidence confirming this hypothesis has been accumulated (*2, 3*). For a direct experimental assault, insulin was chosen as the test protein, because its passage into the blood stream is reflected by the blood sugar level.

Early attempts to administer insulin through the gastrointestinal tract have been reviewed by Jensen (*4*). It is interesting to note that Murlin and Hawley (*5*) and Eaton and Murlin (*6*) used blood plasma as a source of "antitrypsin," whereas Harned and Nash (*7*) used an extract from *Ascaris*. The quantities of the inhibitor present in such preparations were, however, much lower than those used now. The maximal positive effect reported was a temporary disappearance of glycosuria in depancreatized dogs, with (*6*) or without a significant (*7*) lowering of the blood sugar level.

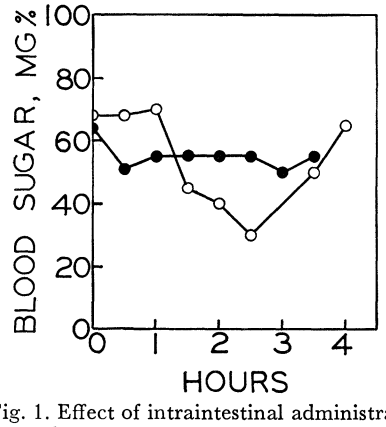

Fig. 1. Effect of intraintestinal administration of insulin on the blood sugar level. Open circles, experiments in which 6 units of insulin (40 units/kg) plus 40 mg of pancreatic inhibitor were injected. Solid circles, control experiments, in which 6 units of insulin (40 units/kg) (no inhibitor) were injected.

A systematic study of different trypsin inhibitors has revealed striking differences with respect to their susceptibility to peptic digestion (*2*) and to their ability to inhibit chymotrypsins (*8*). When these properties were taken into account, only colostrum inhibitor and pancreatic inhibitor were indicated for further study. Pancreatic inhibitor was more easily obtained and thus was used. Once-crystallized inhibitor was prepared according to the method of Kunitz and Northrop (*9*) from "fraction E" (*10*). The regular zinc insulin used was a commercial product (*11*).

Male Sprague-Dawley rats, weighing about 150 g each, were fasted overnight and were anesthetized with Pentothal (thiopental sodium, 40 mg/kg of body weight). The solutions to be investigated were mixed and injected into a loop of jejunum 20 cm long, ligated on both ends. Blood was obtained by clipping off the tip of the tail. Glucose content was determined by the Nelson-Somogyi method (*12*).

Ten experiments in which insulin and inhibitor were injected together were performed. In all ten, a significant drop in blood sugar was observed. Figure 1 illustrates the experiment in which the lowest, and Fig. 2, that in which the highest, dose was used. In other experiments, intermediate doses were used. Ten control experiments were performed by injecting insulin without inhibitor (Figs. 1 and 2); all results were negative. Two control experiments in which the inhibitor alone, and an additional experiment in which insulin plus an excess of protamine, was used, also gave negative results. None of the ten experimental animals died of insulin shock. The highest dose (Fig. 2) produced an effect approximately equivalent to 8 units/kg injected intraperitoneally, suggesting that, at the most, 3 percent of the injected insulin was absorbed.

Substitution of soybean inhibitor for pancreatic inhibitor, in amounts equivalent with respect to trypsin inhibiting power, resulted in very small and nonuniform responses. Since about 80 percent of each inhibitor remained in the loop after 4 hours of exposure, the difference cannot be ascribed to the instability of soybean inhibitor but suggests that pancreatic inhibitor partially protects insulin against destructive agents other than trypsin, whereas soybean inhibitor does not.

It had not yet been established that pancreatic inhibitor protected insulin from destruction. Inactivation in vivo occurred too fast for convenient measurements—that is, in the presence of 40 mg of inhibitor, of 35 units of insulin injected into the loop, only 5 percent was recovered after 3 minutes and less than 1 percent after 30 minutes; the absence of inhibitor did not influence the recovery of insulin after a short exposure, and barely a trace was recovered after 30 minutes. It was decided, therefore, to measure the rate of destruction of insulin in vitro,

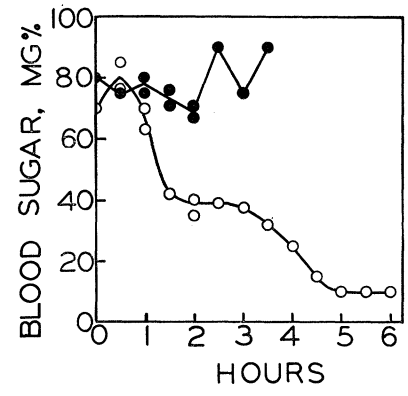

Fig. 2. Effect of intraintestinal administration of insulin on the blood sugar level. Open circles, experiments in which 35 units of insulin (250 units/kg) plus 100 mg of pancreatic inhibitor were injected. Solid circles, control experiments in which 35 units of insulin (250 units/kg) (no inhibitor) were injected.

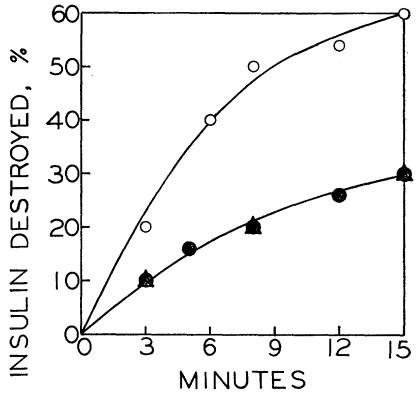

Fig. 3. Rate of destruction of insulin in vitro. Enzymes for the top curve (○) were obtained by injecting into a jejunal loop 1 ml of saline, allowing it to remain 10 minutes, excising the loop, and combining the contents with a 0.5-ml saline washing. Enzymes for the bottom curve (●) were obtained by the same procedure, except that saline containing 40 mg of pancreatic inhibitor per milliliter was used. The incubation mixture consisted of 0.4 ml of enzyme, 2.6 ml of saline containing 0.01*M* phosphate (*p*H 7.3), and 1 ml of insulin, 80 units/ml, at temperature of 37°C. At indicated times aliquots were withdrawn and diluted. In our control experiments, subcutaneous injection of 0.6 units/kg decreased the blood sugar level 35 to 45 percent, when the 1-, 2-, and 3-hour values were averaged and expressed as a percentage of the zero time value. Only dilutions of the in vitro enzyme-insulin mixtures which led to responses in this range were used to calculate percentage of inactivated insulin. Solid triangle, enzymes A to which pancreatic inhibitor was added before the addition of insulin.

and to slow the rate by dilution of the enzymes. The results are presented in Fig. 3 and show that inclusion of pancreatic inhibitor decreased the rate of insulin destruction.

The hypothesis that trypsin inhibitor is of physiological significance in facilitating the intestinal absorption of proteins (insulin) has been confirmed by a direct experiment (*13*).

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Iproniazid Treatment and Metabolism of Labeled Epinephrine in Schizophrenics

Previous work in this laboratory (*1, 2*) showed that when epinephrine labeled with carbon-14 in the beta position was infused into schizophrenic patients and normal subjects, essentially all of the radioactivity was recovered in the urine. When epinephrine labeled with carbon-14 in the methyl group of the side chain was infused, approximately one-third of the radioactivity was recovered in the urine. In both cases, the excretion of biologically active material represented only 1 to 5 percent of the infused epinephrine. A total of 14 infusions were performed. The urine of patients infused with beta-labeled epinephrine was selectively extracted and subjected to paper chromatographic analysis. A major radioactive metabolite was obtained, which possessed the solubility properties of a phenolic acid and had the same R_f values as authentic 3-methoxy-4-hydroxymandelic acid (*3–5*). This radioactive metabolite could not be demonstrated in the urine of patients infused with epinephrine labeled with carbon-14 in the methyl group of the side chain (*2*).

These data suggest the following hypotheses concerning the metabolic transformations of epinephrine: (i) The beta carbon atom remains attached to the benzene ring, and (ii) approximately two-thirds of the molecules of epinephrine lose the methyl group of the side chain. If one assumes that the methyl group of the side chain is lost, together with the amino group, under the influence of amine oxidase, then iproniazid treatment should result in more molecules of epinephrine retaining their methyl groups in the side chain. If this is the case, then more radioactivity should be recovered in the urine of patients receiving iproniazid and infused with methyl-labeled epinephrine.

Three female, chronic schizophrenic patients were placed on iproniazid, 100 mg/day, on 20 June 1957. The dosage was increased to 150 mg/day on 12 August. The first patient was infused with 0.5 mg of methyl-labeled *dl*-epinephrine on 3 September, the second on 18 September, and the third on 9 October. Fifty-nine, 74, and 63 percent of the infused radioactivity was recovered in the urine of these three patients, respectively. This is in contrast to 34 ± 3 percent recovered in the urine of four non-iproniazid-treated schizophrenic patients infused with the same amount of methyl-labeled *dl*-epinephrine. Both types of patients demonstrated typical cardiovascular responses to the infused epinephrine.

Two to 3 weeks after the cessation of iproniazid treatment, the first and second patients were again infused with methyl-labeled epinephrine. Fifty and 43 percent of the infused radioactivity was recovered in the urine of these two patients, respectively. This indicates that approximately half of the effect of iproniazid on monamine oxidase activity, as reflected by the metabolism of exogenously administered epinephrine, was still evident 2 to 3 weeks after the cessation of iproniazid therapy. Thus, approximately twice as many molecules of infused epinephrine retain the methyl group of the side chain when the patient is under iproniazid treatment in the dosages mentioned above as when he is not. These three patients varied in their psychiatric responses to iproniazid therapy. Nevertheless, all three patients showed a remarkably similar alteration in the metabolism of exogenously administered epinephrine.

The question then arose whether the increase in number of molecules retaining the methyl group following iproniazid treatment represents nondegraded, biologically active epinephrine or a stage in metabolism prior to amine oxidase action. Recently, Axelrod (*6*) reported the presence of methoxyepinephrine in the urine of rats, which was found in a greater amount following the intraperitoneal administration of iproniazid and epinephrine.

The following experiments were performed in our laboratory. The urine from patients was collected following the infusion of either beta-labeled or methyl-labeled epinephrine. The urine samples were lyophilized and stored at 0 to 5°C. The lyophilized urine was reconstituted with water and extracted for phenolic acids, according to the procedure of Armstrong *et al.* (*4*). The extracts were concentrated down to a small volume, *in vacuo*, at 45°C. An aliquot of the concentrated extract was chromatographed in the butanol : acetic acid : water system (4:1:5). Another aliquot was chromatographed in the two-phase solvent systems of Armstrong *et al.* (*4*). The phenolic acids were visualized by spraying with diazotized *p*-nitroaniline reagent. Autoradiograms were made from the chromatograms, in order to visualize these metabolites, which were derived from the infused labeled epinephrine. The urine which had been extracted for phenolic acids was hydrolyzed and selectively extracted for methoxyepinephrine in accordance with the procedures outlined by Axelrod (*6*). The extracts were concentrated down to a small volume, *in vacuo*, at 45°C and subjected to paper chromatographic analysis, as outlined above.

The following results were obtained. The urine of non-iproniazid-treated patients infused with beta-labeled epinephrine consistently showed a major radioactive metabolite, which was a phenolic acid having the same R_f value as authentic 3-methoxy-4-hydroxymandelic acid. Very little methoxyepinephrine could be extracted from the urine of these patients. The urine of iproniazid-treated patients infused with methyl-labeled epinephrine consistently showed a major radioactive metabolite, which was a phenolic amine having the same R_f value as authentic methoxyepinephrine (*6*). The increase in excretion of radioactivity by the ipronized-treated patients infused with methyl-labeled epinephrine could be accounted for by the accumulation of methoxyepinephrine with a decrease in formation of 3-methoxy-4-hydroxymandelic acid.

The autoradiograms of urine obtained from patients infused with beta-labeled epinephrine showed the presence of another phenolic acid metabolite of epi-