β-NAPHTHYL-AZO-POLYSTYRENE-INSULIN AS A MEANS OF PROTECTING INSULIN MOLECULE FROM DIGESTIVE ENZYMES.

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SUMMARY

Insulin was bound to diazopolystyrene quenched with β-naphthol in the manner of physical adsorption. The β-naphthyl-azo-polystyrene-insulin was found to interfere with peptic and α-chymotryptic digestions *in vitro* and, when administered into the stomach, produced a hypoglycemic response in rabbits. The degree of hypoglycemic effect of β-naphthyl-azo-polystyrene-insulin was related to the dose of insulin between 20 and 150 units / kg.

INTRODUCTION

Following the discovery of insulin and establishment of its value in the treatment of diabetes mellitus, many investigators explored the possibility of developing an effective method for oral administration. Oral administration of insulin, however, has not been found satisfactory (Tocus *et al.*, 1965), even though a number of recent investigations, either *in vitro* by use of the everted sac technic or *in vivo*, has indicated that insulin can be absorbed from the small intestine of mammals in the presence of proteolytic inhibitors (Laskowski *et al.*, 1958; Danforth *et al.*, 1959; Inouye *et al.*, 1962; Speth *et al.*, 1963). The failure to obtain a hypoglycemic effect when insulin is administered orally therefore attributed to the fact that insulin is rapidly destroyed or inactivated by digestive enzymes.

This report deals chiefly with the possible use of insulin adsorbed physically on β-naphthyl-azo-polystyrene particles for oral administration of insulin. Oral administration of β-naphthyl-azo-polystyrene-insulin to

rabbits resulted in a hypoglycemic response, by protecting insulin from digestive enzymes.

MATERIALS AND METHODS

Preparation of β-naphthyl-azo-polystyrene-insulin: Amberlite XAD-2 resin, a styrene-divinylbenzene copolymer, was purchased from Rohm and Haas Co.. Diazotized poly-p-aminostyrene was prepared by the nitration of polystyrene in H_2SO_4-HNO_3 mixtures, followed by reduction with $SnCl_4$-HCl and diazotization with excess N $NaNO_2$ (Yagi et al., 1960). Diazotized poly-p-aminostyrene was treated with excess β-naphthol to block the diazo groups. After being washed with borate buffer, pH 8.0, to remove excess β-naphthol, the filter cake was mixed with insulin solution at pH 8.0 for overnight with a gentle stirring (weight ratio of β-naphthyl-azo-polystyrene (β-NAS) to insulin 5 : 1) After being washed with borate buffer to remove excess insulin, the wet cake was lyophylized and stored at 5°C. The insulin content of β-naphthyl-azo-polystyrene-insulin (β-NAS-I) was determined by an amino acid autoanalyzer (Hitachi, Japan).

Determination of biological potency of β-naphthyl-azo-polystyrene-insulin:

The six male rabbits weighing from 2.0 to 2.5 kg were injected intravenously with β-NAS-I suspension at a dose of 1 unit / 0.5 ml / kg. The blood glucose lowering effect of β-NAS-I in rabbits was compared with that of standard insulin. Blood glucose determination was made by the method of Somogyi-Nelson.

Hydrolysis of β-naphthyl-azo-polystyrene-insulin with pepsin and α-chymotrypsin:

β-NAS-I containing 10 mg of insulin was incubated for 3 hours at 37°C with a purified pepsin (weight ratio of substrate to enzyme 200 : 1) in 0.2M citrate buffer, pH 2.2 and with a purified α-chymotrypsin (weight ratio of substrate to enzyme 10 : 1) in 0.2 M borate buffer, pH 8.0. Samples from the digestion mixtures were withdrawn at 0, 20, 90 and 180 minutes, and stopped the enzyme reaction by the addition of 5% trichloroacetic acid. After

centrifugation, free amino acid residues liberated from β-NAS-I were determined by ninhydrin method.

Experimental animal: Male rabbits weighing from 2.0 to 2.5 kg. were starved for 16 to 18 hours. Twenty to one hundred and fifty units of β-NAS-I (calculated from its biological potency) were dissolved in borate buffer, pH 8.0. Doses of insulin in a volume of 1 ml / kg were infused directly into the stomach through a stomach tube. Blood was drawn from the marginal ear vein in rabbits at 0, 30, 60, 90, 120 and 180 minutes.

RESULTS AND DISCUSSION

The insulin content of β-NAS-I preparation used in this experiment was found to be 103 mg / g of β-NAS-I. The biological potency of β-NAS-I was found to be 40 to 50% of the original value. The firmness of the binding of insulin to β-NAS was tested by successive washing with borate buffer, pH 8.0, followed with acetate buffer, pH 2.3. A small amount of insulin was eluted by borate buffer washing without any appreciable loss of biological potency. The biological potency, on the contrary, was reduced to 10 to 20% of the original value after liberating about one third of insulin in β-NAS-I by mild acid treatment. These findings suggest that insulin fixed tightly on β-NAS plays no significant role in its pharmacodynamic action. Insulin fixed loosely appears to be of importance for its bilogical potency.

It has been well known that crystalline pepsin, trypsin and α-chymotrypsin destroyed the activity of insulin. Under mild conditions, trypsin did not destroy the activity of insulin (Nicol, 1960). The peptic and α-chymotryptic hydrolyses of insulin and β-NAS-I were further studied in the condition described. During the incubation of insulin with pepsin at pH 2.2, a small amount of precipitates was appeared, when the ninhydrin values began to fall after reaching maximum. To confirm the identity of these precipitates, the precipitates were collected by centrifugation and analyzed by an amino acid autoanalyzer. Amino acid analysis of these precipitates revealed

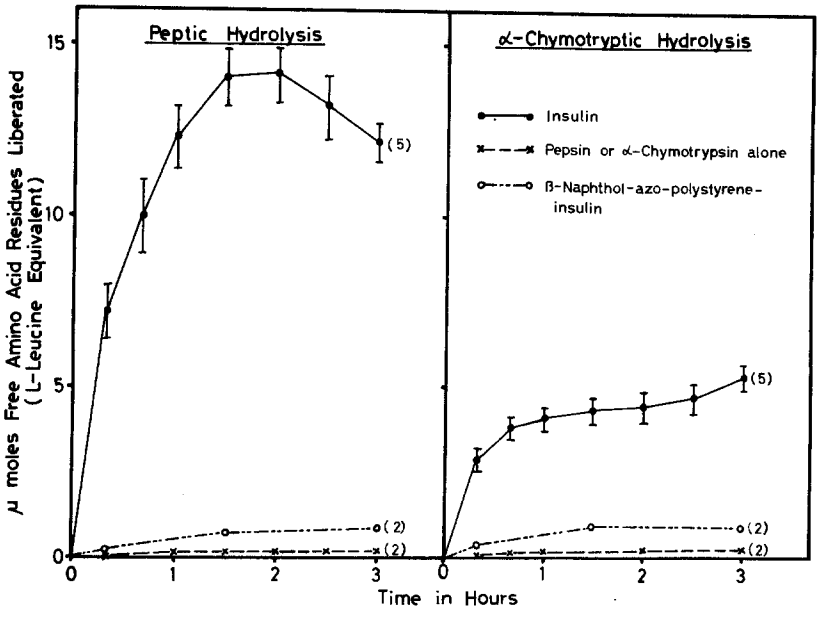
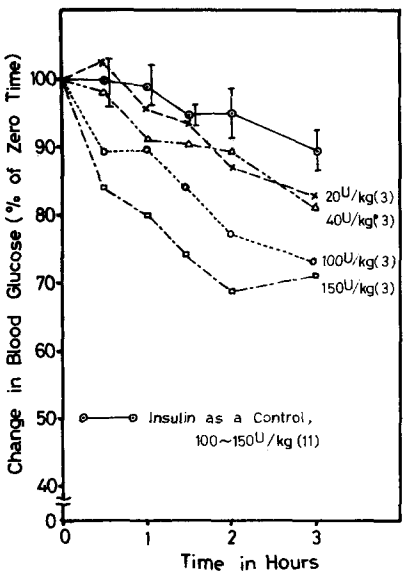


Fig. 1. The rate of hydrolysis of β-naphthyl-azo-polystyrene-insulin by pepsin and α-chymotrypsin. The results are expressed as μ moles amino acid residues liberated from 10 mg insulin (L-leucine equivalent). The results are given as means ± standard errors with number of incubations indicated in parenthesis.

significant quantities of valine, glutamic acid, asparatic acid, glycine and leucine. The results could not, of course, decide whether these precipitates were simple amino acids or small fragment peptides. As shown in Fig. 1, the destruction of insulin by pepsin was found to exceed that by α-chymotrypsin greatly. The degree of hydrolysis of β-NAS-I, as measured by the ninhydrin method, on the other hand, was decreased markedly. It has not yet been established, however, that insulin adsorbed on β-NAS could be protected from digestive destruction since inactivation in vivo occured too fast and to too great an extent.

The effect of oral administration of β-NAS-I through a stomach tube in rabbits is summarized in Fig. 2. Infusion of insulin solutions (up to 150 units / kg) into the stomach failed to produce a significant decrease in blood glucose levels. A significant depression of the blood glucose was noted with infusion of 100 and 150 units / kg of β-NAS-I into the stomach.



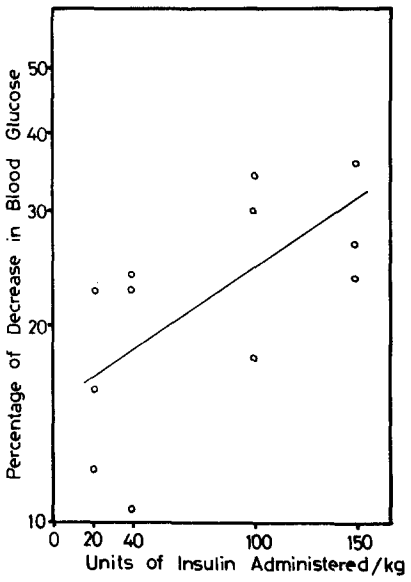


Fig. 2. (Left). Changes in blood glucose following a single oral administration of β-naphthyl-azo-polystyrene-insulin to rabbits. Insulin preparations were infused through a stomach tube. The number of animals is given in parentheses ; the results with control insulin are given as means ± standard errors.

Fig. 3. (Right). The degree of decrease in blood glucose as a function of dose of β-naphthyl-azo-polystyrene-insulin. Blood glucose values before and three hours after dosing were compared. The results are expressed as percentages of decrease in blood glucose from pre-dose value.

In each experiment, it is apparent that the maximal effect was obtained in the first two to three hours after the infusion was completed. The degree of hypoglycemic effect of β-NAS-I was related to the dose of insulin between 20 and 150 units / kg. (Fig. 3).

Previous reports have indicated intestinal absorption of insulin in the presence of dyes (Murlin et al., 1940), saponins (Lasch et al., 1927) or proteolytic inhibitors such as diisopropylfluorophosphate (Danforth et al., 1959), soybean or pancreatic trypsin inhibitor (Laskowski et al., 1958; Danforth et al., 1959) and trasyrol (Inouye et al., 1962). Recently the possible use of water-in-oil-water (W/O/W) emulsions (Engel et al., 1963) or egg yolk emulsions (Kelker, 1966) has been reported as a means of facilitating gastrointestinal absorption of normally non-absorbed water soluble biopolymers. In these experiments, however, insulin was infused directly into jejunum and

ileum. The hypoglycemic effect of insulin, when administered into the stomach, has been demonstrated only in suckling mammals (Mosinger et al., 1959; Kelly, 1960).

Our results reported here suggest a possible means of protecting insulin from digestive enzymes. β-NAS-I, when administered into the stomach, was found to produce a comparable depression of the blood glucose. Further studies are necessary to elucidate the nature of insulin adsorbed on carrier particles as well as routes of administration.

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