# Enzymatic Hypoglycemia in Alloxan Diabetic Rats.* (30229)

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The enzymes alpha-chymotrypsin and trypsin exhibit insulin-like activity toward the isolated rat diaphragm(1). Their enzymatically inactive diisopropylfluorophosphate derivatives appear to have lost this property (1). Since the accepted criterion for the action of insulin in the body is a fall in concentration of blood sugar, it became of interest to know whether the insulin-like enzymes could lower blood sugar. Insulin is itself endowed with esterolytic(2) and proteolytic activity(3); the present study is therefore part of a larger effort to determine if relationships exist between proteolytic and insulin-like activity on one hand, and hormonal action and enzymatic activity on the other.

*Methods and procedure.* Male rats of the Holtzman strain, weighing 160-180 g, were made diabetic with intraperitoneal injection of 150 mg/kg alloxan. Blood samples were taken from the tail veins of fed rats under light ether anesthesia immediately after administration of the enzyme, and at 2-hour intervals thereafter. Twice-crystallized trypsin and chymotrypsin and their diisopropylfluorophosphate derivatives were purchased from Worthington Biochemical Corp. They were dissolved in 0.154 M sodium chloride and were injected intraperitoneally as single doses of 146 mg/kg. Chymotrypsin was treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) as described by Schoellmann and Shaw(4). Blood sugar was measured by Nelson's modification(5) of the Somogyi method.

*Results and discussion.* When chymotrypsin or trypsin is administered to fed diabetic rats, a dose of 146 mg/kg administered intraperitoneally decreases blood sugar significantly (Fig. 1 & 2). Similar doses of their enzymatically(8) inactive diisopropylfluorophosphate derivatives (DIP) are incapable of hypoglycemic action (Fig. 1 & 2). The hypoglycemic action of the native enzymes is not unexpected since both trypsin and chymotrypsin enhanced the uptake of nonmetabolizable sugars by rat diaphragm and stimulated the synthesis of glycogen from glucose there(1). The lack of hypoglycemic action of DIP-enzymes is also in accord with the results of the earlier study(1).

Both trypsin and chymotrypsin are inhibited by diisopropylfluorophosphate because of reaction of this organophosphate with a unique serine residue in the same tetrapeptide sequence(9,10). This is the serine which is acylated as an intermediate in the catalytic mechanism(11). In this context it is significant that insulin itself is acylated as an intermediate when the hormone catalyzes the hydrolysis of nitrophenyl esters(2).

Recently the irreversible inactivation of chymotrypsin by L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) was shown to be caused by alkylation of the active site histidyl residue in position number 57 of the enzyme's B chain(4,6,7). This provided the first direct evidence for the involvement of histidine in the catalytic mechanism of chymotrypsin. In the present experiments, chymotrypsin was inactivated with an 18-fold molar excess of TPCK(4). Loss of enzymatic activity was ascertained by a failure to catalyze the hydrolysis of acetyl-L-tyrosine ethyl ester(9). Administration of the TPCK-derivative of chymotrypsin at 146 mg/kg intraperitoneally to alloxan diabetic rats proved ineffective in lowering the blood sugar significantly (Fig. 3).

Trypsin and chymotrypsin are fundamentally similar in primary structure, mechanism of action, and biological properties(12). To the latter category may now be added their insulin-like activity. The results of the present study indicate that these insulin-like enzymes act like insulin only as long as they are functional enzymes. This property is dependent on the integrity of the active serine and the active histidine which is alkylated by

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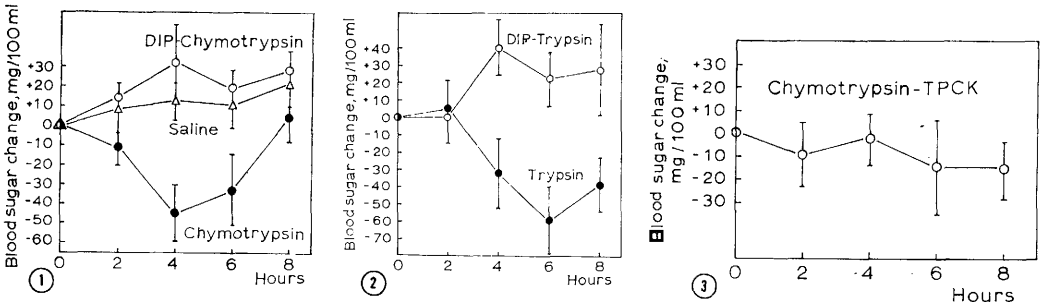


FIG. 1. Effect of chymotrypsin on blood sugar of alloxan diabetic rats. Each point represents the mean value for 6 rats, and vertical bars indicate standard error of mean.

FIG. 2. Effect of trypsin on blood sugar of alloxan diabetic rats. Each point represents the mean value for 6 rats, and vertical bars indicate standard error of mean.

FIG. 3. Effect of chymotrypsin-TPCK on blood sugar of alloxan diabetic rats. Each point represents the mean value for 6 rats, and vertical bars indicate standard error of mean.

TPCK. Since serine and histidine are at the active sites of trypsin and chymotrypsin, and since covalent derivatives of these residues are neither catalytically nor hypoglycemically functional, it is likely that enzymatic reactions and hypoglycemia are catalyzed by the same mechanism. We may then speak of an enzymatic, or catalyzed, hypoglycemia. The question as to where in the body trypsin and chymotrypsin catalyze the insulin-like effect has already been answered: they stimulate cellular uptake of sugar and conversion of glucose to glycogen in muscle(1). The latter action is presumably an effect on the transglucosylase enzyme(1). How trypsin and chymotrypsin act on the muscle cell membrane to render it more permeable to glucose is open to speculation. One possibility is that the membrane barrier to glucose is removed by the hydrolysis of peptide bonds at the cell surface. This would facilitate an action of the proteases on the intracellular glycogen synthetic system. The proteases might actually enter the cells. Chymotrypsin is known to be able to cross cell barriers(13). Either inside the cell, or by triggering a chain of events from the outer surface, trypsin and chymotrypsin might alter cellular ultrastructure in such a way as to bring synthetic systems into more favorable relations to their substrates than in the absence of these stimulants. This is an idea advanced some years ago by Krahl(14) to account for the action of insulin on cells.

The use of trypsin and chymotrypsin as hypoglycemic agents may permit a critical evaluation of these proteins as insulin models. The recent elucidation of the complete amino acid sequence of chymotrypsin(15) opens the way to further structural comparisons with insulin. In the light of the proteolytic nature of insulin, these comparisons as well as active site studies are capable of yielding predictions which may be tested.

*Summary.* Trypsin and chymotrypsin, administered intraperitoneally as single doses of 146 mg/kg to fed diabetic rats, are able to lower blood sugar. Covalent derivatives of active-site serine in both enzymes, and of active-site histidine in chymotrypsin, are hypoglycemically inactive. Trypsin and chymotrypsin produce hypoglycemia as a result of their actions in stimulating cellular uptake of sugar and conversion of glucose to glycogen in muscle. The recent demonstration of the proteolytic nature of insulin, and the complete elucidation of the amino acid sequence of chymotrypsin allow the formulation of predictions concerning the mechanism of action of insulin.

1. Rieser, P., Rieser, C. H., Proc. Soc. Exp. Biol. and Med., 1964, v116, 669.

2. Hartley, B. S., Kilby, B. A., Biochem. J., 1954, v56, 288.

3. Rieser, P., Rieser, C. H., Biochem. Biophys. Res. Comm., 1964, v17, 373.

4. Schoellmann, G., Shaw, E., Biochem., 1963, v2, 252.

5. Nelson, N., J. Biol. Chem., 1944, v153, 375.

6. Meloun, B., Popísilová, D., Biochim. Biophys. Acta, 1964, v92, 152.

7. Ong, E. B., Shaw, E., Schoellmann, G., J. Biol. Chem., 1965, v240, 694.

8. Schwert, G. W., Takenaka, Y., Biochim. Biophys. Acta, 1955, v16, 570.

9. Schaffer, N. K., May, C. S., Jr., Summerson, W. H., J. Biol. Chem., 1953, v202, 67.

10. Dixon, G. H., Kauffman, D. L., Neurath, H., ibid., 1958, v233, 1373.

11. Oosterbaan, R. A., Van Adrichem. M. E., Biochim. Biophys. Acta, 1958, v27, 423.

12. Walsh, K. A., Neurath, H., Proc. Nat. Acad. Sci., U. S., 1964, v52, 884.

13. Avakian, S., Clin. Pharmacol. Therap., 1964, v5, 712.

14. Krahl, M., The Action of Insulin on Cells, Academic Press, New York, 1961.

15. Hartley, B. S., Nature, 1964, v201, 1284.

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## Na and K Bound to an Ether Soluble Compound in Myocardium of Renal Hypertensive Dogs.* (30230)

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Sodium metabolism has been suspected of being deranged in the disease state hypertension. The evidence for this is the effectiveness of a low sodium diet in clinical hypertension (1) and the use of desoxycorticosterone and high NaCl intake to produce experimental hypertension(2).

Such a derangement of sodium metabolism has not been readily shown by methods of tissue analysis. In our laboratory we have been interested in the fact that sodium is contained in an ether extract of tissues, presumably bound to an ether soluble compound. This study is to determine whether the amounts of this compound might be altered in the myocardium of hypertensive compared with normotensive dogs.

*Methods. Animals.* Adult dogs, 11 male and 1 female, 18 to 28 kg, were maintained on a standard commercial diet. The mean blood pressure was measured 2 to 3 times a week by femoral artery puncture with a 20-gauge needle, until a constant control pressure was obtained. The control mean blood pressures were from 95 to 130 mm Hg. The dogs were then subjected to the Grollman procedure to produce renal hypertension. In the Grollman procedure a figure-of-eight ligature is placed around one kidney; 2 weeks later the contralateral kidney is removed. All surgery was carried out under pentobarbital anesthesia (30 mg/kg).

After recovery from surgery the blood pressure was measured weekly or biweekly until hypertension became established or until it was apparent that hypertension would not develop. Hypertension was considered present when the mean blood pressure was maintained at 40 mm Hg above preoperative levels. The mean blood pressure in dogs with hypertension ranged from 160 to 175 mm Hg. The dogs were sacrificed by anesthetizing with pentobarbital and rapidly opening the chest and removing the heart for samples. Triplicate samples were taken from each ventricle.

Of the 11 dogs subjected to the Grollman procedure 5 developed hypertension. Six dogs showed no rise in blood pressure with blood pressures between 90 to 130 mm Hg. These and one sham operated animal were considered controls.

*Analytical technique.* The samples of myocardium (1 to 3 g) were frozen. Later they were thawed and subjected to an extraction procedure for ether soluble compounds containing sodium and for ether soluble compounds containing potassium (ESC-Na and ESC-K)(3). Previous studies in the laboratory revealed no changes in these components due to storage in the frozen state. Tissues from a control dog were analyzed at the same time as tissues from a hypertensive

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