EFFECTS OF INSULIN ON THE STRUCTURE AND METABOLISM OF GLYCOGEN IN CHEMICALLY-INDUCED DIABETIC RATS

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INTRODUCTION

IT HAS BEEN generally accepted that insulin plays an essential role in the regulation of metabolism particularly that of carbohydrate. Insulin, when administered, favours an increase in the activities of specific enzymes concerned with glucose uptake, glycogen synthesis, fat synthesis, and growth. Its primary function is to favour the formation and storage of substances of large molecular weight such as glycogen, fats, and proteins.

In the study of control of glycogen synthesis, *in vivo* glycogenic procedures, such as administration of D-glucose to starved animals, have been used. Chapman *et al.*, (1955) observed differences in the iodine-adsorption characteristics between the rapidly formed liver-glycogen and 'normal' liver-glycogens. It was suggested that these differences were due to structural differences between the two types of glycogen. It is our aim to investigate the possibility further by rendering the animals diabetic and further treating them with insulin so that the normal and newly formed glycogens can be isolated and their structures compared by both iodine-staining and enzymic degradation methods.

Alloxan and streptozotocin have been the widely used agents, in recent years, to induce diabetes in laboratory animals. Their diabetogenicity arises from its cytotoxic effect on pancreatic islet B-cells and B-cell necrosis has been consistently demonstrated in all species of animals rendered diabetic by these agents. However, streptozotocin, for instance, caused no change in pancreatic glucagon content in the rat (Pagliara *et al.*, 1975) and led to predominance of A_2-cells in the guinea pig islets (Petersson *et al.*, 1970).

In the present study the effects of alloxan-or streptozotocin-induced diabetes and of insulin treatment of these diabetic rats on liver glycogen metabolism have been examined. The enzymes involved in the regulation of glycogen synthesis, as well as in its degradation, have been examined in three groups of animals, i.e. control, alloxan-or streptozotocin-induced-diabetic and insulin-treated diabetic animals. These studies also explore the possible mechanism of insulin action on glycogen synthesis by studying the structure of glycogen. The structure will reflect the activities of the enzymes involved in glycogen synthesis.

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MATERIALS AND METHODS

Alloxan monohydrate was obtained from Hopkin & Williams, England. Streptozotocin was obtained from I.C.N. Pharmaceuticals, England. Protamine zinc insulin (40 units/ml) was obtained from Weddel Pharmaceuticals, London.

Bacillus subtilis alpha amylase and sweet potato beta-amylase in ammonium sulphate suspension were obtained from Sigma Chemical Company, U.S.A.

Experimental Procedures With Animals

The experimental animals were fed on a high carbohydrate diet-pellets (obtained from Zuellig Feed Mills, Malaysia) and 5% glucose solution.

Male rats (*Rattus norvegicus*) aged between two and three months were obtained from the Central Animal House, Faculty of Medicine, University of Malaya.

Control animals were starved for 48 h and then fed with 5% glucose solution. They were maintained on the high carbohydrate diet until sacrifice.

Experimental animals were made diabetic by a daily intraperitoneal injection of streptozotocin (55 mg/kg body weight in 0.01M sodium citrate of pH 4.5) or alloxan (150 mg/kg). They were maintained daily on a high carbohydrate diet. The onset of diabetes was monitored every 24 h after alloxan/streptozotocin treatment by estimating glucose in the urine using Clinistix strips (Ames Co., Australia). After the fifth day, the rats were all found to be diabetic. These diabetic animals were divided into two groups. One group received no further treatment. The other group was used for insulin treatment. This group of diabetic animals was given an intramuscular injection of protamine zinc insulin (40 units/kg body weight) daily before sacrifice. Throughout the experimental period, they were maintained on the high carbohydrate diet. The rats were sacrificed at 0, 2, 4, 6 and 8 days after insulin treatment. A time lapse of 2 h after insulin administration was allowed before the sacrifice since insulin activation of the glycogen synthetase enzyme would have been completed by this time period (Gold, 1970).

Extraction and Purification of Glycogen

Immediately after sacrifice, the liver was removed, frozen and stored at -20^{o} for enzyme assays. One gramme of the liver was used for the extraction of glycogen using the Pfluger method as described by Manderson *et al.*, (1968). Pieces of liver were digested in 40% (w/v) potassium hydroxide (5 ml) on a boiling water bath. After 45 min, the digest was cooled and two volumes of ice-cold ethanol were added. The glycogen was allowed to precipitate overnight at 4^{o}.

After centrifugation, the precipitated glycogen was redissolved in a known volume of water and aliquots were taken for the determination of carbohydrate by the phenol-sulphuric acid method (Dubois *et al.*, 1956).

The glycogen was purified by five reprecipitations with ethanol, washed twice with boiling ethanol, and finally dried with ether.

This purified glycogen was used for structural studies using iodine-staining, alpha-amylolysis and beta-amylolysis techniques.

Iodine-Staining Procedure

The iodine-staining procedure used was that described by Archibald *et al.*, (1961). The spectrum between 400-600 nm of a solution containing 0.2% of glycogen, 0.2% of iodine, and 2.0% of potassium iodide in half-saturated ammonium sulphate was recorded against an iodine-iodide control using a Beckman Acta III spectrophotometer. From the spectrum, the wavelength of maximum absorption (λ_{max}) was determined. Under these conditions, the average chain-length of the glycogen (c.l.) is given by the relationship:

$$\text{c.l.} = 16 + 0.114\,(\lambda_{max} - 500)$$

Alpha-Amylolysis Procedure

Digests containing glycogen (0.5 mg), alpha-amylase (60 units), and 0.1% sodium chloride in a final volume of 4 ml were incubated at 37%for 12 h under an atmosphere of toluene. After incubation, the production of reducing sugars was determined as maltose by the Nelson-Somogyi method (Robyt and Whelan, 1968) and the total carbohydrate was determined by the phenol-sulphuric acid method. The apparent percentage conversion into maltose (alpha-amylolysis limit) is related to the average chain-length under these conditions by the expression:

$$100/\text{c.l.} = 23.4 - 0.20\ (\text{alpha-amylolysis limit})$$

Beta-Amylolysis Procedure

Digests containing glycogen (1 mg) and beta-amylase (60 units) in 0.1 ml of 0.1 M sodium acetate buffer (pH 4.8) were incubated at 37%for 12 h under an atmosphere of toluene. After incubation, the percentage conversion into maltose (beta-amylolysis limit) was determined in a similar manner to the determinations of alpha-amylolysis limit. The average, exterior chain-length (e.c.l.) was calculated from the average chain-length determined by alpha-amylolysis and the beta-amylolysis limit, by the expression:

$$\text{e.c.l.} = \frac{\text{c.l. x beta-amylolysis limit}}{100} + 2.5$$

The average interior chain-length (i.c.l.) was calculated from the expression:

$$\text{i.c.l.} = \text{c.l.} - (\text{e.c.l.} + 1)$$

Enzyme Studies

The following enzyme activities were studied using the methods as indicated: hexokinase/glu-

cokinase (Leloir and Trucco, 1955), phosphoglucomutase (Hers, 1964), glucose-6-phosphatase (Hers, 1964), succinate dehydrogenase (Slater and Bonner, 1952) and phosphorylase (Hers, 1964).

RESULTS

Rats were divided into three groups of six animals. The first group was treated as the control and received no treatment. Of the two remaining categories of diabetic animals, one group received daily administration of insulin. The results shown are an average taken from three separate experiments. Table I shows the variation in liver-glycogen content at the end of the eighth day under different experimental conditions.

It can be seen that the onset of diabetes lowered the liver glycogen content and that there was a rapid resynthesis of glycogen with insulin administration.

The results showed that there was no significant difference between the 'insulin-treated' and 'control' glycogens but there appeared to be a small decrease in the average chain-lengths of the 'diabetic' glycogen.

Fig. 1 shows the variation in hexokinase/glucokinase activities with alloxan/insulin administration time. The insulin-treated rats showed an increase in the enzyme activities whereas the diabetic group showed a decrease in enzyme activities.

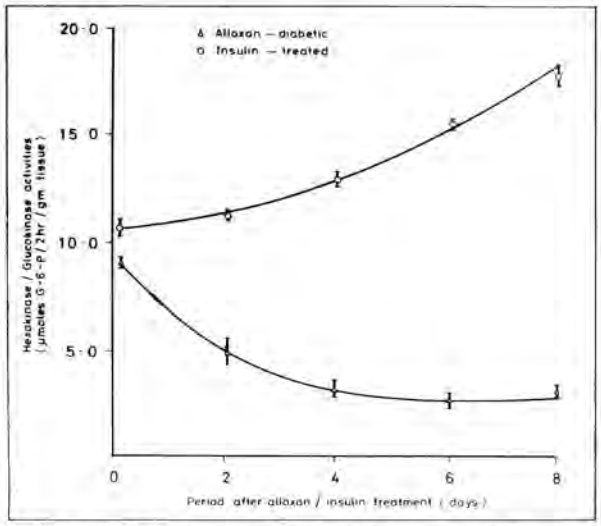

Fig. 1. Effect of the successive administration of alloxan/insulin on the hexokinase/glucokinase activities.

TABLE I

Structural Analysis Of Glycogens From Control, Diabetic And Insulin-Treated Animals

Treatment	Liver glycogen content (% wet) weight)	λ_{max}	c.l. $_{av}$	e.c.l. $_{av}$	i.c.l. $_{av}$
Control	3.4±0.2	466±1	12.1±0.4	7.3±0.2	3.8±0.1
Diabetic	2.9±0.3	457±4	11.1±0.5	6.6±0.4	3.5±0.2
Insulin-treated.	5.5±0.5	469±3	12.5±0.2	7.6±0.5	3.9±0.1

Although both groups of animals showed increased phosphoglucomutase activities, insulin-treated ones demonstrated a higher enzyme activity (Fig. 2).

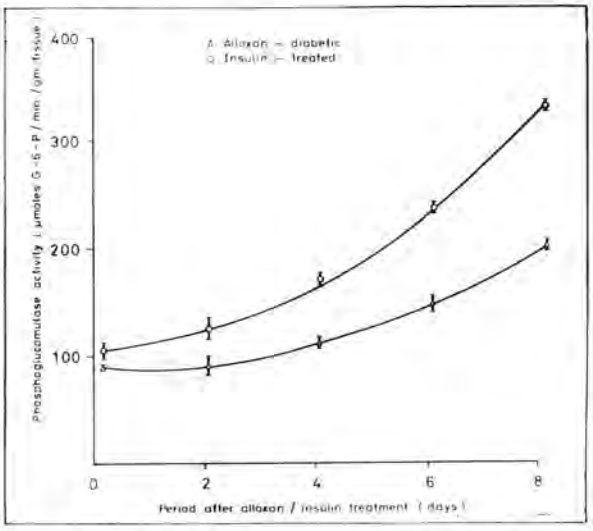

Fig. 2. Effect of the successive administration of alloxan/ insulin on the phosphoglucomutase activity.

For the glucose-6-phosphatase activity both groups of animals showed an initial decrease (Fig. 3). The enzyme activity later continued to rise in the diabetic rats but decreased in the insulin-treated group.

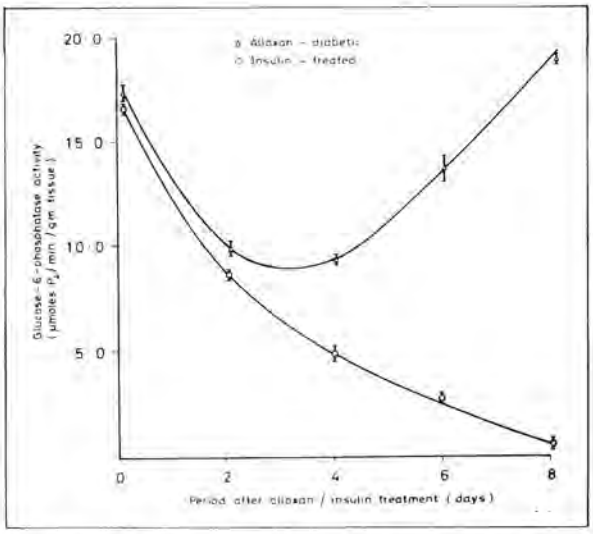

Fig. 3. Effect of the successive administration of alloxan/ insulin on the glucose-6-phosphatase activity.

A tricarboxylic acid cycle enzyme, namely, succinate dehydrogenase was studied (Fig. 4).

Both groups of animals showed an increase in the enzyme activities.

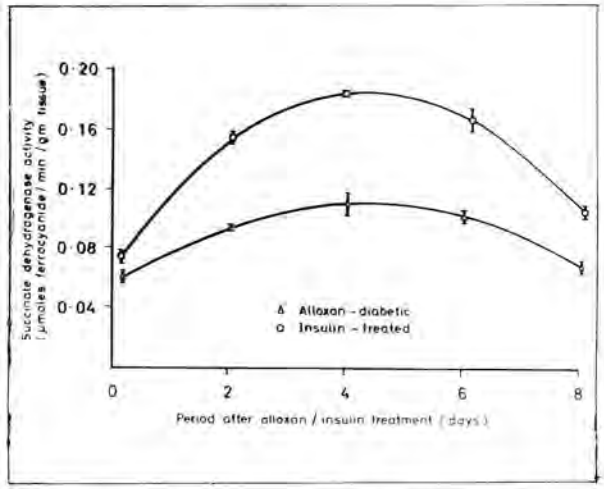

Fig. 4. Effect of the successive administration of alloxan/ insulin on the succinate dehydrogenase activity.

The insulin-treated animals showed a greater decrease in the phosphorylase activity than found in the diabetic group (Fig. 5).

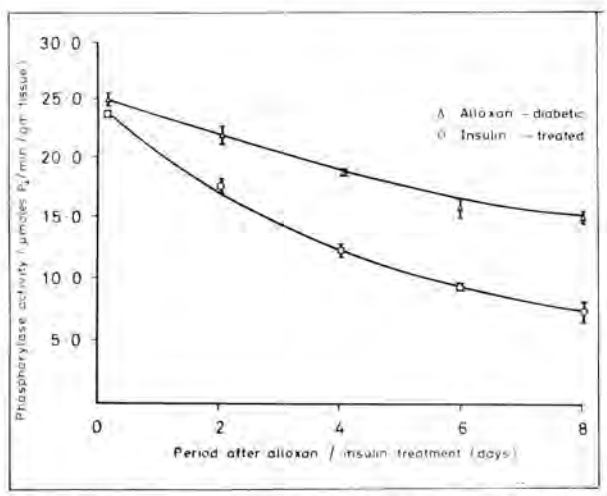

Fig. 5. Effect of the successive administration of alloxan/ insulin on the phosphorylase activity.

DISCUSSION

The variation in chain-lengths found in the groups of animals (Table 1) is probably caused by the relative activities of three enzymes, namely, glycogen synthetase, phosphorylase and branching enzyme.

Our results show that insulin does increase glycogen synthesis as evidenced by the larger amount of glycogen content in the insulin-treated animals than found in the untreated ones. Thus, one of the effects of insulin is to increase the glycogen content by activating the glycogen synthesising systems.

The fact that the glycogen structure is little altered in the insulin-treated animals when compared with the control group (although the glycogen content is different in both cases) indicates that the glycogen synthesising enzymes, such as glycogen synthetase, branching enzyme, etc. are all involved in the increase in glycogen content. The branching enzyme must also play a role in the glycogen synthesis as a linear molecule of glycogen would result if glycogen synthetase alone was involved.

In diabetes, glycogen storage is reduced and this is reflected in the decreased kinase activities during glycogenesis (Fig. 1).

Fig. 2 shows that the higher phosphoglucomutase activity in the insulin-treated animals is caused by insulin.

The fact that the glucose-6-phosphatase activity rises, after an initial fall, in the diabetic rats but decreases further in the insulin-treated group is significant (Fig. 3). In diabetes, gluconeogenesis is increased and glucose-6-phosphatase is a key gluconeogenic enzyme.

There appears to be a distince relationship between the patterns of enzymic activities and accumulation of glycogen in the rats. Phosphoglucomutase, one of the enzymes necessary for synthesis of glycogen, increases in activity during deposition of glycogen. In contrast, activity of glucose-6-phosphatase, which mediates a reaction competitive to glycogenesis, decreases concomitantly. Therefore, accumulation of glycogen in the liver does not seem to be solely a result of a lack of activity of glucose-6-phosphatase but rather is a consequence of interplay in activity of enzymes involved in glycogen synthesis and degradation.

The increase in the succinate dehydrogenase activity of the diabetic animals is due to an increase in succinate (Fig. 4). This is due to the fact that, in diabetes, proteins and fats are rapidly catabolized and they add their intermediates into the tricarboxylic acid cycle. For the insulin-treated animals, the increase in the enzyme activity was possibly due to increased glucose oxidation in tissues. This increased oxidation can be achieved by the glycolytic pathway, the hexose monophosphate shunt or the tricarboxylic acid cycle.

Insulin provokes inactivation of phosphorylase (Fig. 5) and possibly phosphorylase kinase as well. The question how insulin causes the inactivation of phosphorylase kinase cannot be readily answered. We do not know if the presence of glucagon or other effectors in the portal blood is required for this insulin effect.

A sequential inactivation of phosphorylase and activation of glycogen synthetase has been observed in monkeys treated with glucose or with insulin (Curnow *et al.*, 1975). The sequential change in the activities of the two enzymes has been explained by the observation that phosphorylase *a* strongly inhibits glycogen synthetase phosphatase. It appears therefore that the profound inhibition of glycogen synthetase phosphatase by phosphorylase *a* is a general control mechanism of glycogen metabolism in the liver.

Insulin could act by lowering the hepatic concentration of cyclic AMP. But, it has been found by van de Werve *et al.*, (1977) that insulin did not change the activity of the cyclic AMP dependent protein kinase. Since neither the concentration of cyclic AMP nor the activity of protein kinase were affected by insulin, it appears likely that the hormone acted through a messenger different from cyclic AMP, possibly a cation. Whether calcium ions play a role in the activation of the glycogen synthetase and glycogen phosphorylase phosphatases (Thambyrajah and Karunairatnam, 1972), or whether the insulin effects are exerted by the alterations in the substrates for phosphoprotein phosphatase are not known and further studies are required to unravel the mechanisms of insulin action.

SUMMARY

The structure and metabolism of liver glycogen were studied in rats fed on a high carbohydrate diet and rendered diabetic by alloxan/streptozotocin administration and from the diabetic rats further injected with insulin. The structures of these liver-glycogens isolated, were determined using enzymic and iodine staining techniques. The glycogen content increased in the insulin-treated group whereas that of the diabetic group decreased. The glycogen structure from both groups of animals when compared with that of the control group appeared to be similar. Insulin increased the activities of enzymes such as hexokinase/glucokinase, phosphoglucomutase, and succinate dehydrogenase. It also caused a decrease in glucose-6-phosphatase and phosphorylase activities. Possible mechanisms of insulin actions on glycogen synthesis were also explored.

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