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THE ACTION OF HYPOGLYCAEMIC SULPHONYLUREAS ON CARBOHYDRATE METABOLISM IN THE FASTED RAT

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Tolbutamide and carbutamide given orally to fasted rats cause a rise in the liver glycogen content 1½ to 3½ hr. after administration of the drugs. Glycogen accumulates preferentially in the right lobe. Subcutaneously injected tolbutamide has the same effect. Both sulphonylureas cause inhibition of glucose-6-phosphatase activity of rat liver homogenates *in vitro*, but at drug concentrations comparable with those found in plasma of treated patients the degree of inhibition is less than 10%. Livers from treated rats show normal glucose-6-phosphatase activity. The glucose uptake of the isolated rat diaphragm is unaffected by the sulphonylureas added *in vitro*. Diaphragms from treated rats show normal glucose uptake in the presence or absence of insulin. The inferences to be drawn from these results are discussed in the light of previous work. It is concluded that the sulphonylureas exert their hypoglycaemic action by inhibiting glycogenolysis and it is suggested that they might do so by inhibiting release of glucagon from the pancreas.

Various theories have been put forward to explain the hypoglycaemic action of the sulphonylureas, N-(*n*-butyl)-N'-(*p*-aminophenyl)-sulphonylurea (BZ55 or carbutamide) and N-(*n*-butyl)-N'-(*p*-methylphenyl)-sulphonylurea (D860, Rastinon or tolbutamide). These theories, summarized recently by Levine and Duncan (1956), have suggested the following possible modes of action: 1. Stimulation of insulin production in the β cells of the pancreas. 2. Inhibition of insulin destruction in the liver, and hence potentiation of its action. 3. Inhibition of glucagon production in the pancreas. 4. Suppression of glycogen breakdown in the liver by inhibition of one or other of the enzymes of the glycolytic system. 5. Inhibition of gluconeogenesis. Much experimental evidence has been quoted in favour of each of these hypotheses, but none of them has been generally accepted. Essentially, they postulate that the sulphonylureas affect either the central mechanisms in the liver controlling carbohydrate metabolism or the utilization of carbohydrate in the peripheral tissues. The experiments to be described were designed to determine in which of these two most likely sites the sulphonylureas exert their main action.

METHODS

Rats.—Male albino Wistar rats weighing 130 to 230 g. were fed *ad libitum* on a standard rat cake diet. They were then starved for 24 hr., but had free access to water. At the end of this time they were weighed and killed by decapitation.

Sulphonylureas.—For oral administration with a stomach tube, carbutamide was suspended in water, and tolbutamide dissolved in 3% aqueous sodium carbonate. Tolbutamide was also given subcutaneously in 10% aqueous solution (*p*H 8.9). For either route of administration the drugs were given in a dose of approximately 500 mg./kg. body weight, the animals being killed 1½, 2½, or 3½ hr. later. Control animals received 3% sodium carbonate (2 ml.) orally, or physiological saline (2 ml.) subcutaneously, whichever was appropriate.

Blood Sugar Estimation.—The blood sugar concentrations of all rats were determined in samples obtained from the neck at the time of decapitation. A modification of the Schaffer-Hartmann method as described by King (1951) was used throughout for glucose estimation.

Rat Diaphragm.—Glucose consumption by the isolated diaphragm was determined by the method described by Vallance-Owen and Hurlock (1954), with the exception that Krebs-Ringer bicarbonate buffer was used as the medium for soaking and incubating the muscle instead of the buffer described by Gey and Gey (1936). Glucose consumption during incubation for 90 min. is expressed as μg. glucose/100 mg. dry weight of muscle.

Liver Glycogen Estimation.—Either the whole liver or portions (0.5 to 1.0 g.) of the right median and left lobes were removed and placed in hot 30%

aqueous KOH within 60 sec. of the time of decapitation. The glycogen content, as determined by the method of Good, Kramer, and Somogyi (1933), is expressed as g. glucose/100 g. wet weight of liver, or as total liver content in mg. glucose/100 g. of rat body weight.

Glucose-6-phosphatase Activity.—This was estimated by a modification of Swanson's method (1950). Immediately after death the liver was removed and cooled rapidly to approximately −10°, such tissue being used immediately or kept frozen at −15° for subsequent use up to two weeks later; activity was found to be unaffected by storage under these conditions. Portions (0.3 to 0.5 g.) were homogenized in 20 vol. of ice-cold 1% aqueous glucose solution, and the homogenates used immediately. Glucose-6-phosphate solution (0.04 M) was obtained by shaking 80 mg. of the barium salt (L. Light) with 2 ml. of an ion-exchange resin (Zeo-Karb 225, sodium form) suspended in 5 ml. of maleate buffer (0.1 M, *p*H 6.3) for 10 min. Tolbutamide and carbutamide, when added *in vitro*, were dissolved in the maleate buffer before addition to the incubation mixture.

To 0.3 ml. of the liver homogenate and 0.6 ml. of the maleate buffer, with or without added sulphonylurea, was added 0.3 ml. of glucose-6-phosphate solution. Using the method originally described by Ernster, Zetterström, and Lindberg (1950), and later modified by Strickland, Thompson, and Webster (1956), the inorganic phosphate content of this mixture was determined immediately after addition of the glucose-6-phosphate and again after 30 min. incubation at 37°. Glucose-6-phosphatase activity is expressed as mg. inorganic P released/g. wet weight of liver/30 min.

RESULTS

Rat Diaphragm.—If the sulphonylureas have any direct peripheral action one would expect to be able to show an effect *in vitro* upon glucose consumption by the isolated rat diaphragm. When added to the incubation medium in concentrations of 50 mg./100 ml., neither tolbutamide nor carbutamide exerted any appreciable effect (Table I); glucose consumption by one hemidiaphragm incubated in the presence of the drug was compared with that of its partner which was used as a control. Clarke, Davidson, Schonbaum, and Serman (1956) had suggested that glucose consumption in the rat diaphragm might be inhibited by carbutamide. The present results are not in agreement with their conclusions and contraindicate any direct peripheral action by these drugs; on the other hand, they do not eliminate the possibility that the sulphonylureas act indirectly on peripheral tissues by increasing insulin secretion *in vivo*. To test this latter possibility tolbutamide was given 2 hr. before the animals were killed, but again glucose consumption by the diaphragms of treated rats incubated in the presence or absence of insulin (10^{-3} i.u./ml.) was the same as that of the controls (Table II).

TABLE I

EFFECTS OF TOLBUTAMIDE AND CARBUTAMIDE (50 MG./100 ML.) ON GLUCOSE CONSUMPTION OF THE ISOLATED RAT DIAPHRAGM

Mean values ± standard error.

Drug	No. of Rats	Glucose Consumption μg./100 mg. Dry Wt.	
		Control Hemidiaphragm	Test Hemidiaphragm
Carbutamide ..	8	263±6	244±19
Tolbutamide ..	11	323±9	359±12
,, ..	8	289±12	274±23

TABLE II

EFFECT OF TOLBUTAMIDE (500 MG./KG.) 2 HR. BEFORE DEATH UPON GLUCOSE CONSUMPTION OF THE ISOLATED RAT DIAPHRAGM

Mean values ± standard error.

	Glucose Consumption (μg./100 mg. Dry Wt. of Muscle)	
	Hemidiaphragm Without Insulin	Hemidiaphragm + Insulin 10^{-3} i.u./ml.
Controls (7) ..	303±13	612±33
Tests (8)	310±13	649±9

That the sulphonylureas do not act peripherally is also suggested by the fact that, in a few experiments which were carried out to study the action of tolbutamide *in vivo*, no rise in muscle glycogen was found 2½ hr. after administration of the drug; this is in accordance with the results of Miller and Dulin (1956) and of Beringer and Lindner (1956).

Liver Glycogen. — Several workers have described the effect of tolbutamide upon the glycogen content of the liver. Bander and Scholz (1956) noted that it had a sparing action on liver glycogen in fasting guinea-pigs, a finding which Tybergheim, Halsey and Williams (1956) and Lang and Sherry (1956) confirmed in rats. Miller and Dulin (1956) observed a rise in liver glycogen content in fasted rats killed 8 hr. after tolbutamide, and a similar effect was produced (Beringer and Lindner, 1956) in fasted rabbits 2½ hr. after carbutamide.

These reports suggest that the sulphonylureas might lower the blood sugar by inhibiting glycogenolysis in the liver. If this is so, the rise in liver glycogen might be expected to occur in the first few hr. after administration of the drugs, in close relation to the fall in blood sugar. Of the experiments quoted above, only those of Beringer and Lindner (1956) refer to this early period. In order to investigate this point further, rats were killed at intervals in the first 3½ hr. after a standard dose of the sulphonylurea.

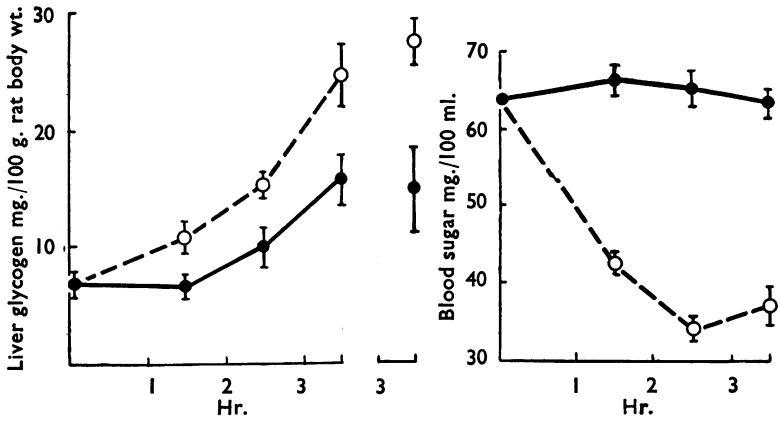

FIG. 1.—Glycogen content of livers and blood sugar levels in starved rats after treatment with oral tolbutamide (500 mg./kg.). Except where whole livers were used, the glycogen content was determined from the mean of the concentrations in portions of the right median and left lobes. Solid circles, control rats. Open circles, test rats. The points standing alone for the glycogen content at 3½ hr. were obtained from whole livers. Mean values with ± standard error are plotted.

Glycogen content was estimated using either whole livers or approximately equal portions from the right median and left lobes. When such portions were taken, it was assumed that the glycogen concentration in the liver as a whole was equal to the mean of the concentrations in the two portions examined. This assumption is supported by the evidence quoted by MacIntyre, Pedersen, and Maddock (1941) and borne out in the present experiments in which no difference was found between the concentrations in the two lobes of livers of control rats (Fig. 2). In the test animals, however, the distribution of glycogen in the two lobes was unequal, a discrepancy which is discussed below.

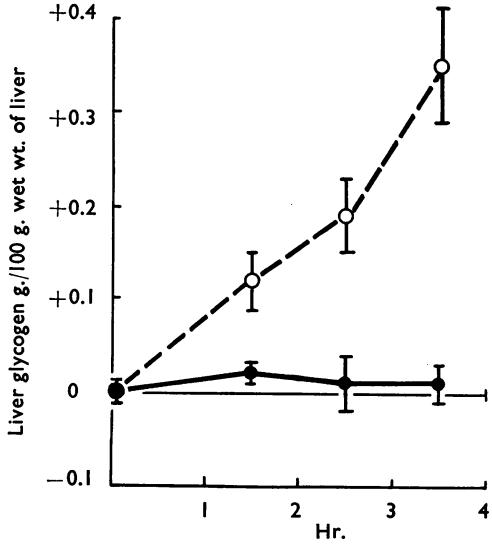

FIG. 2.—Difference in glycogen contents of liver lobes after oral tolbutamide (500 mg./kg.). Solid circles, control rats. Open circles, test rats. Mean values with ± standard errors are plotted.

Fig. 1 shows the glycogen content of the liver and the blood sugar levels at intervals after oral tolbutamide. It can be seen that in control animals given 3% sodium carbonate alone there was an appreciable rise in liver glycogen from an initial fasting level of 7.1 ± 1.0 mg./100 g. rat body weight to a value of 15.7 ± 2.3 mg./100 g. rat body weight 3½ hr. later. After oral tolbutamide, however, the rise in liver glycogen was significantly greater. Thus, when whole livers were examined, the glycogen content 3½ hr. after the drug was found to be 27.3 ± 1.9 mg. compared with 14.7 ± 3.5 mg./100 g. rat body weight in the untreated animals. Similar figures were obtained when portions of the right median and left lobes were used and the glycogen content of the whole liver calculated from the mean of the concentrations in the two portions. In test animals the value was 24.4 ± 2.8 mg./100 g. compared with 15.7 ± 2.3 mg./100 g. rat body weight in controls. Using the latter method of estimation this rise in liver glycogen was found to occur as early as 1½ hr. after oral tolbutamide. Carbutamide, when given orally, produced a comparable effect (Table III).

When portions were analysed it became apparent that the glycogen concentrations in the two lobes from treated animals were unequal. The concentration in the right median lobe was consistently higher than that in the left, the difference increasing with time to a value of 0.35 g./100 g. wet wt. of liver 3½ hr. after the administration of the drug (Fig. 2). At all times the glycogen content of the right median lobe from treated animals was significantly greater than that in the corresponding lobe from controls; the difference in the left lobes was much less marked (Fig. 3).

During the 3½ hr. period after oral tolbutamide, therefore, the liver glycogen content of fasted rats rises at a much greater rate than that of control

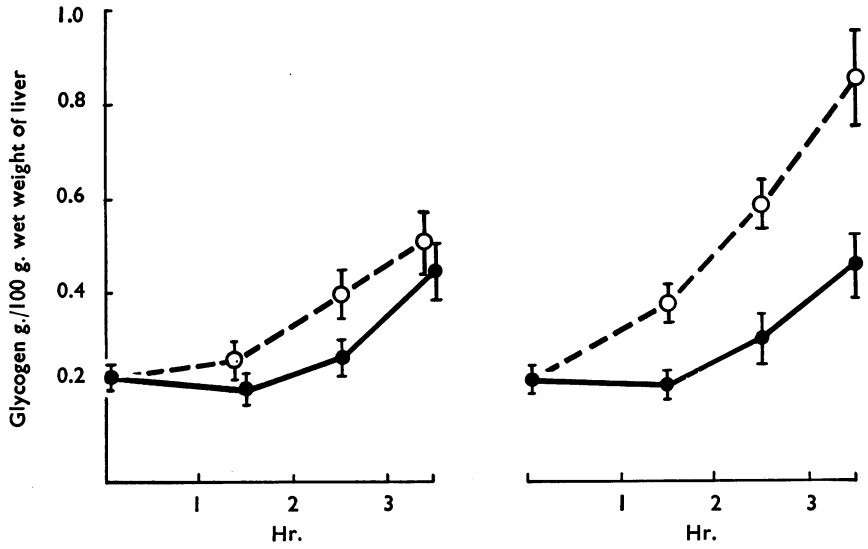

FIG. 3.—Glycogen content of liver lobes after oral tolbutamide (500 mg./kg.). The estimations from the left lobe are plotted on the left and those from the right median lobe on the right. Solid circles, controls. Open circles, test animals. Mean values with ± standard errors are plotted.

animals ; it does so asymmetrically and it does so early in the hypoglycaemic period.

It was thought possible that the asymmetrical distribution of glycogen found in the livers of treated rats might coincide with an asymmetrical distribution of the drug after its absorption from the alimentary tract due to " streamlining " in the portal circulation (Himsworth, 1950). This possibility was ruled out when it was found that the same asymmetrical distribution of glycogen occurred in animals given tolbutamide by subcutaneous injection (Table III).

Glucose-6-phosphatase.—Hawkins, Ashworth, and Haist (1956), Tybergheim *et al.* (1956), and Ashmore, Cahill, and Hastings (1956) have all demonstrated inhibition of glucose-6-phosphatase activity by carbutamide and tolbutamide *in vitro* ; Vaughan (1956), on the contrary, was unable to do so. Direct inhibition of this enzyme *in vivo*, occurring at an early stage after administration of the drug, could explain its hypoglycaemic action. To test this point glucose-6-phosphatase activity was compared in livers from normal and treated rats. Homogenates of livers from normal fasted rats (34 experiments) showed a mean glucose-6-phosphatase activity of 6.8 ± 0.2 mg. P/g. wet weight of liver/30 min.; fasted rats given carbutamide 2 hr. before killing, or tolbutamide 1 hr. or 2½ hr. before death, showed liver glucose-6-phosphatase activity well within the normal range. This finding confirms the results obtained by Ashmore *et al.* (1956). If the drugs were to inhibit glucose-6-phosphatase *in vivo* by forming an inactive enzyme-drug complex, homogenization of the livers of treated rats in 20 vol. of 1% glucose might be expected to cause dissociation of such a complex, so that the homogenates, as shown above, would exhibit normal activity. In further experiments, therefore, livers of normal and treated rats were homogenized in 1% glucose containing tolbutamide (50 mg./100 ml.) and were incubated in the presence of the drug in the same concentration. Under these conditions it was again found that the livers of normal and treated rats had the same enzyme activity. When, however, carbutamide or tolbutamide was added *in vitro* to the incubation mixture, inhibition of

TABLE III

LIVER GLYCOGEN CONTENT OF FASTED RATS 2½ HR. AFTER ORAL AND SUBCUTANEOUS SULPHONYLUREAS

Mean values ± standard error.

Drug	Route of Administration	No. of Rats	Glycogen g./100 g. Wet Weight of Liver		Total Liver Glycogen mg./100 g. Body Weight
			Left Lobe	Right Median Lobe	
Control	Oral	8	0·27±0·04	0·31±0·05	9·9±1·6
,,	Subcutaneous	6	0·22±0·02	0·24±0·02	6·4±2·0
Tolbutamide	Oral	15	0·40±0·05	0·59±0·04	15·3±1·2
,,	Subcutaneous	14	0·37±0·04	0·74±0·06	19·1±2·0
Carbutamide	Oral	12	0·49±0·04	0·59±0·05	17·0±1·5

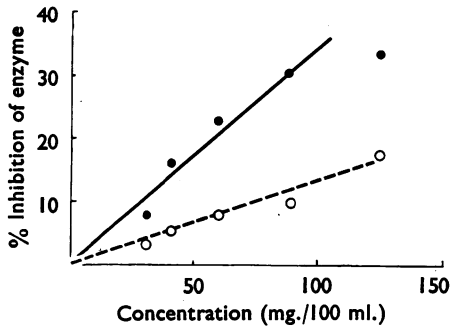

FIG. 4.—Inhibition *in vitro* of glucose-6-phosphatase by sulphonylureas. Solid circles, tolbutamide. Open circles, carbutamide. The points are mean values.

enzyme activity was detected and increased in a linear manner as the drug concentration was raised (Fig. 4). Using drug concentrations similar to those likely to occur in the blood during successful therapy (10 to 20 mg./100 ml.) very little inhibition of glucose-6-phosphatase occurred *in vitro*. Even with tolbutamide in a concentration of 125 mg./100 ml., which is close to the maximum solubility at *p*H 6.3 (Forist and Chulski, 1956), only 30 to 40% inhibition was produced.

DISCUSSION

A fall in the concentration of glucose in the blood and interstitial fluids could be due to a rise in the rate of consumption of glucose in the tissues, or to a fall in the rate of release of glucose from the liver. The results described above suggest that, as the blood sugar level falls under the influence of carbutamide or tolbutamide, there is a concomitant rise in the glycogen content of the liver, but no change in the rate of glucose consumption in the peripheral tissues. It seems unlikely, therefore, that the sulphonylureas act by stimulating the secretion of insulin, or by inhibiting its destruction. Further, the rapid rise in liver glycogen does not support the hypothesis that they act primarily by inhibiting gluconeogenesis, but suggests rather that they act by inhibiting glycogenolysis.

Glycogen is synthesized and broken down in several stages, which are shown in Fig. 5. It seems unlikely that accumulation of glycogen could result from the inhibition of any enzyme such as phosphoglucomutase, which has an action that is completely reversible. On the other hand, inhibition of any enzyme concerned solely with glycogen breakdown could produce a fall in blood sugar concentration associated with a rise in liver glycogen content; glucose-6-phosphatase and the " debranching " enzyme fall into this category. It should also be mentioned that although liver phosphorylase catalyses a reversible reaction, Cahill, Ashmore, Zottu, and Hastings (1957) have recently reported experiments which suggest that the predominant direction of this reaction can be influenced *in vitro* by sodium and potassium ions and by glucagon and adrenaline. Even under ionic conditions which favour glycogenolysis least (high potassium and low sodium concentrations), glucagon *in vitro* has a marked glycogenolytic effect. It is, therefore, possible that inhibition of phosphorylase itself could lead to inhibition of glycogenolysis.

Accumulation of glycogen in the livers of patients suffering from one form of glycogen storage disease is accompanied by a reduction in glucose-6-phosphatase activity of the liver, hypoglycaemia and an impaired response to the hyperglycaemic action of exogenous adrenaline and glucagon (Andersen, 1952).

The results outlined above suggest that the accumulation of liver glycogen found in rats treated with tolbutamide is not due to glucose-6-phosphatase inhibition. In this respect they support the clinical findings of Moorhouse and Kark (1956), Fajans, Louis, Seltzer, Johnson, Gittler, Hennes, Wajchenberg, Ackerman and Conn (1956) and Butterfield, Camp, Hardwick and Holling (1957), who, among others, have shown that the response of treated patients to exogenous adrenaline and glucagon is normal.

Although it seems unlikely that these drugs act by inhibiting glucose-6-phosphatase, there remains the possibility that they might inhibit the breakdown of glycogen to glucose-1-phosphate either directly or indirectly. The action of the sulphonylureas upon the release of glucose from liver slices

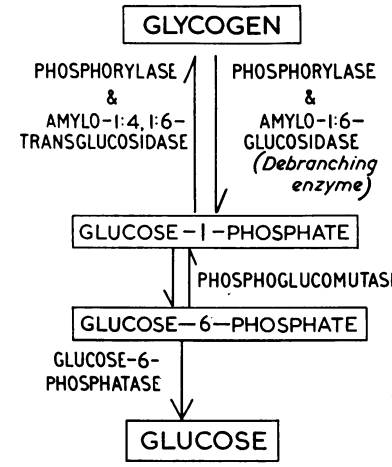

FIG. 5.—Mechanism of glycogenolysis in the liver.

has been studied by several workers (Clarke *et al.*, 1956; Berthet, Sutherland, and Makman, 1956; Vaughan, 1956; Mohnike and Knitsch, 1956). The results of these experiments are conflicting, but do not suggest that the drugs act by inhibiting the phosphorylase mechanisms directly. They could, however, act indirectly by inhibiting the release of hormones or other substances which stimulate this reaction, such as adrenaline and glucagon, both of which are known to stimulate it *in vivo* and *in vitro*.

Houssay and Penhos (1956) and Lang and Sherry (1956) showed that adrenalectomized animals are exceedingly sensitive to tolbutamide, and that hypoglycaemia develops even if cortical hormones are given concurrently. Grant (personal communication) has shown that in a rabbit with a denervated ear, hypoglycaemia produced by intravenous tolbutamide is accompanied by release of endogenous adrenaline, which produces a normal hyperglycaemic effect. In the adrenalectomized rabbit no such adrenaline response occurs after the drug and the blood sugar falls until convulsions develop, yet exogenous adrenaline in such a rabbit exerts its normal hyperglycaemic effect. This evidence indicates that sulphonylureas do not interfere to any appreciable extent with the release or action of adrenaline *in vivo*, a conclusion which is supported by clinical findings.

It is not possible, on present evidence, to explain the accumulation of liver glycogen which occurs soon after the sulphonylureas are administered to rats. Bertram, Bendfeldt, and Otto (1955) and Anderson, Perfetto, Termine, and Monaco (1956) have suggested that these drugs might interfere with the release of glucagon from the pancreas; they are not likely to interfere with the action of glucagon for the reasons already given. Glucagon, according to Cahill *et al.* (1957), exerts a glycogenolytic action upon the phosphorylase reaction, so that a reduction in the amount of glucagon reaching the liver could result in a decrease in the rate of glycogenolysis and hence an accumulation of liver glycogen. The asymmetrical distribution of this accumulated glycogen cannot be explained and it is not apparently due to uneven distribution of the drug in the liver following absorption from the alimentary tract; oral and parenteral tolbutamide produce the same effect.

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