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Effects of glucose and fructose on conjugation of *p*-nitrophenol in hepatocytes of normal and streptozotocin-diabetic rats

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The effects of experimentally induced diabetes on the hepatic monooxygenase system have generally been studied in isolated microsomes [1–3], and there has been little interest in the effects of diabetes on drug metabolism in the intact hepatocyte. This is despite potentially significant interactions between intermediary metabolism, which is severely disrupted by diabetes, and monooxygenase pathways. One such interaction is at the level of NADPH [4–6]. It was suggested that diminished availability of reduced pyridine nucleotide in the diabetic liver was responsible for inhibition of oxidative drug metabolism *in vivo* [7, 8]. Conjugation reactions may also be altered as a result of abnormal intermediary metabolism in the diabetic liver [9, 10]. Changes in the concentrations of uridine diphosphate glucuronic acid (UDPGA) and NAD^+ in the diabetic liver have been reported [11, 12], and formation of glucuronide conjugates is quite sensitive to alterations in the levels of UDPGA [13, 14] and NAD^+ [15].

The present studies were undertaken to determine the effects of experimental diabetes on conjugative metabolism in isolated rat hepatocytes, and to determine the effects of manipulating carbohydrate metabolism in hepatocytes of normal and diabetic rats with glucose and fructose.

Methods. Male Sprague–Dawley rats, weighing 175–250 g, were obtained from Flow Laboratories (Dublin, VA). Animals were given free access to Purina Lab Chow and water and were housed in group cages in a constant temperature room (22°) with a 12-hr light–dark cycle. Rats were fasted overnight prior to being killed. Diabetes was induced with streptozotocin (STZ) as described previously [16] and the rats were used in studies 10 days later.

Hepatocytes were prepared as described previously [16], except that glucose was omitted from the incubation buffer. In some experiments the medium was resupplemented with either glucose or fructose prior to incubation. Hepatocytes were counted with a hemocytometer and diluted with incubation buffer to a final concentration of 2–3 × 10^6 viable cells per ml. Viability was judged with trypan blue, which was routinely excluded from >95 per cent of the cells. Hepatocytes (10–25 ml) were incubated at 37° with shaking under an atmosphere of 95% O_2/5% CO_2. Conjugation reactions were initiated by the addition of *p*-nitrophenol (pNP) after 2–3 min of temperature equilibration. Reactions were terminated by removing 6 ml aliquots of incubation mixture and adding them to 0.56 ml of 40% (w/v) trichloroacetic acid, after which the precipitate was sedimented by centrifugation. The supernatant fraction was adjusted to pH 5.0 with 2 N sodium acetate, and conjugated metabolites of pNP were measured after hydrolysis to the phenol by β-glucuronidase and arylsulphatase [15]. Duplicate 1 ml aliquots were combined with 40 μl of β-glucuronidase (200 units) or arylsulphatase (5 units) and another duplicate 1 ml aliquot was used without enzyme addition to determine the level of unconjugated phenol. Samples containing β-glucuronidase were incubated at 37° for 2 hr, and samples with sulphatase for 1 hr. All samples were then alkalinized with 1.4 ml of 1.2 N sodium carbonate and absorbance was measured at 400 nm spectrophotometrically. To determine the quantity of the specific conjugated metabolites, the amount of the free phenol detected in the absence of hydrolytic enzymes was subtracted from values obtained in samples which had undergone hydrolysis.

Arylsulphatase (type H-1, Sigma Chemical Co., St. Louis, MO) was dissolved in 0.2 N sodium acetate, pH 5.0, and β-glucuronidase was purchased as a solution (Glucurase, Sigma) in the same buffer. D-Saccharic acid-1,4-lactone (Sigma) was incorporated into the arylsulphatase solution at a concentration of 20 mM to inhibit β-glucuronidase activity which contaminates this preparation. All other reagents were the same as those used previously [16].

Statistical analyses were performed by analysis of variance with differences between individual means determined by the 5 per cent least significant difference [17].

Results. When incubated in a glucose-free medium, hepatocytes isolated from diabetic rats exhibited a greater capacity to form the glucuronic acid conjugate of pNP than cells from control rats (Fig. 1). The effect was greatest during the initial 5 min of incubation, when liver cells of diabetic rats formed nearly 600 per cent more pNP glucuronide than controls. Administration of insulin to diabetic rats returned glucuronidation to control levels. There was a small but significant increase in pNP sulphate formation by diabetic cells after a 30-min incubation, whereas no differences from control were detected at earlier incubation times.

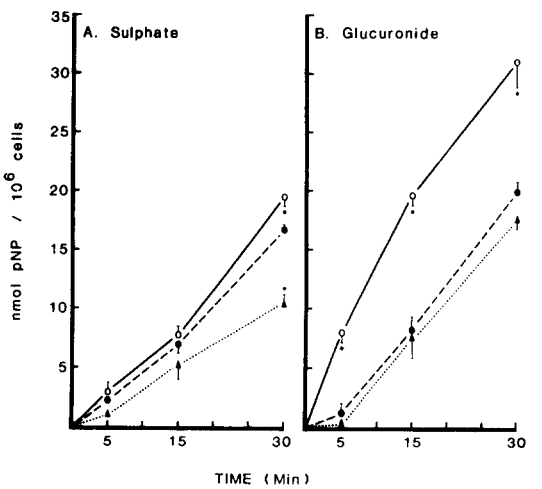

Fig. 1. Sulphate and glucuronide production from pNP as a function of time in hepatocytes of normal, diabetic, and insulin-treated diabetic rats. Animals were killed 10 days after STZ injection, and hepatocytes of control (●–––●), diabetic (○——○), and insulin-treated diabetic (▲···▲) rats were incubated in a glucose-free medium with 100 μM pNP. Protamine Zinc insulin (3 units) was administered on days 5–8. Points represent means ± S.E. of data from four animals. An asterisk (*) indicates a significant change from control, $P < 0.05$.

The differences in pNP glucuronide formation in hepatocytes of normal and diabetic rats were diminished when cells were incubated in the presence of 5.5 mM glucose and were abolished when glucose concentration in the medium was increased to 25 mM (Table 1). This occurred because hepatocytes from control rats responded to the addition of glucose with greater production of pNP glucuronide, whereas hepatocytes of diabetic rats did not. Glucuronidation in hepatocytes of insulin-treated diabetic rats was not different from that of controls, whether in the absence or presence of glucose. Although glucuronidation in hepatocytes of diabetic rats did not respond to the addition of glucose, cells from insulin-treated animals regained the responsiveness seen in control cells. Additional incubations were performed in the presence of fructose at a concentration (10 mM) which maximally stimulated glucose production in hepatocytes of diabetic rats [18]. Fructose actually decreased production of pNP glucuronide in control hepatocytes. The sugar, however, had no effect on cells of diabetic rats or insulin-treated diabetic rats.

Sulphate conjugation in hepatocytes of diabetic rats was greater than in hepatocytes of normal rats in the absence of glucose; however, the differences between the groups were not statistically significant when cells were incubated in the presence of 5.5 or 25 mM glucose (Table 2). Sulphate conjugation in hepatocytes of insulin-treated diabetic rats was less than in hepatocytes of control rats whether incubated in the presence or absence of glucose. The addition of glucose caused small increases in sulphate conjugation in cells of insulin-treated rats; however, it did not affect sulphation in hepatocytes of normal or diabetic rats. Fructose inhibited sulphate conjugate formation in hepatocytes of diabetic rats, but not in cells of control or insulin-treated diabetic rats.

Table 1. Effects of glucose and fructose on the formation of pNP glucuronide in hepatocytes of control, diabetic, and insulin-treated rats*

	pNP glucuronide (nmoles)		
Treatment	Control	Diabetic	Insulin-treated diabetic
None	19.92 ± 0.89	31.02 ± 2.22†	17.51 ± 0.83
5.5 mM Glucose	24.19 ± 1.22‡	33.16 ± 1.70†	25.99 ± 1.69‡
25 mM Glucose	30.94 ± 1.72‡	33.75 ± 3.31	28.46 ± 2.24‡
10 mM Fructose	15.34 ± 0.58‡	29.35 ± 1.80†	18.86 ± 1.84

* Animals were killed 10 days after STZ injection, and insulin-treated rats received 3 units of Protamine Zinc Insulin on days 5–8. Glucose was omitted from the incubation buffer during preparation of hepatocytes, and glucose and fructose were resupplied just prior to the start of incubation. Cells were incubated with 100 μM pNP for 30 min. Values are means ± S.E. of data from four animals.

† Significant change from control hepatocytes incubated under identical conditions, $P < 0.05$.

‡ Significant change from cells incubated without glucose, $P < 0.05$.

Table 2. Effects of glucose and fructose on the formation of pNP sulphate in hepatocytes of control, diabetic, and insulin-treated rats*

	pNP sulphate (nmoles)		
Treatment	Control	Diabetic	Insulin-treated diabetic
None	16.63 ± 0.30	19.45 ± 0.83†	10.33 ± 0.78†
5.5 mM Glucose	16.52 ± 1.07	19.18 ± 1.03	13.34 ± 0.80†‡
25 mM Glucose	17.35 ± 1.21	19.32 ± 0.69	14.18 ± 0.94†‡
10 mM Fructose	14.28 ± 0.62	15.50 ± 0.95‡	10.15 ± 0.95†

* Animals were killed 10 days after STZ injection, and insulin-treated rats received 3 units of Protamine Zinc Insulin on days 5–8. Glucose was omitted from the incubation buffer during preparation of hepatocytes, and glucose and fructose were resupplied just prior to the start of incubation. Cells were incubated with 100 μM pNP for 30 min. Values are means ± S.E. of data obtained from four animals.

† Significant change from control hepatocytes incubated under identical conditions, $P < 0.05$.

‡ Significant change from cells incubated without glucose, $P < 0.05$.

Discussion. We have demonstrated in the present report that glucuronidation of pNP in isolated hepatocytes was enhanced by STZ diabetes. The most notable increase in the rate of glucuronide formation in diabetic cells relative to controls occurred during the initial 5 min of incubation. The effect was probably not a result of more rapid uptake of pNP by hepatocytes of diabetic rats, because sulphation was not similarly affected at the early time. It was also unlikely that enhanced glucuronidation was due to increased glucuronyltransferase activity in diabetic cells, as we have found no such change in enzyme activity assayed in microsomes of diabetic rats (unpublished observation). Enhanced glucuronide formation was more likely related to increased availability of the cofactor UDPGA. It was postulated that the flux of glucose in tissues of diabetic animals was shunted to insulin-insensitive pathways when flow through insulin-sensitive routes was inhibited [19–21]. One outcome of such shunting was increased production of UDPGA, resulting primarily because conversion of UDP-glucose to glycogen was severely limited in the diabetic liver [22]. It has been demonstrated that increased production of UDPGA can augment the capacity of the liver to form glucuronic acid conjugates even without a detectable increase in glucuronyltransferase activity [13] and, in the presence of high substrate concentrations, supply of the cofactor is more important in determining rates of glucuronidation than activity of the enzyme [14].

Rates of glucuronidation in control hepatocytes were higher in the presence of glucose than it its absence. Glucose was presumably phosphorylated by glucokinase and subsequently served to increase the supply of UDPGA. In addition, ATP production from the glycolytic pathway contributed to the generation of UDPGA. In diabetic livers, however, glucokinase activity was reduced by as much as 95 per cent and glucose phosphorylation was inhibited more than 85 per cent [22], so already high rates of glucuronidation were not augmented by glucose in hepatocytes of diabetic rats. As a result, the differences between control and diabetic cells in the capacity to form glucuronides were diminished when 5.5 mM glucose was added to the media and were eliminated when 25 mM glucose further increased carbohydrate flux to UDPGA in control cells.

Fructose had no effect on glucuronidation in hepatocytes of diabetic rats. This was surprising because it has been reported to stimulate maximal production of glucose in these hepatocytes [18]. In addition, fructose conversion to glucose-6-phosphate did not involve glucokinase, so it was expected to increase carbohydrate flux to UDPGA despite inhibition of glucose phosphorylation. In contrast, fructose inhibited glucuronidation of pNP in control hepatocytes. The ketose was reported to decrease ATP concentrations in the liver *in vitro* due to its rapid utilization in the conversion of fructose to fructose 1-phosphate by fructokinase [23, 24]. The formation of UDPGA is dependent upon ATP for phosphorylation of glucose and for rephosphorylation of UDP to UTP. Fructose was shown to decrease the concentration of UDP-glucose in the liver [23, 24], and it presumably decreased UDPGA levels as well. Thus, inhibition of glucuronidation in control hepatocytes by fructose appears to involve a decrease in UDPGA availability as a result of ATP depletion. Depletion of ATP in hepatocytes of diabetic rats probably had less effect on UDPGA levels since its use in the phosphorylation of glucose was already greatly decreased. In addition, fructose was found to stimulate glycogen synthesis in normal livers but not in livers of diabetic rats [25, 26], thereby diverting UDP-glucose from UDPGA synthesis in livers of normal rats.

Sulphation was greater in hepatocytes of diabetic rats than in those of controls when incubated in the absence of glucose. Statistical significance was reached in this case because of low variance; differences of similar magnitude were not significant in incubations with glucose. Sulphate conjugation in cells of insulin-treated rats was less than in those of normal rats. Fructose caused a small decrease in sulphation in hepatocytes of diabetic rats and a small decline which was not of statistical significance in control cells. Decreases in sulphation would be expected to result from depletion of ATP because the nucleotide is utilized in the synthesis of 3′-phosphoadenosine-5′-phosphosulphate, the cofactor required for sulphation.

It was recently demonstrated by Price and Jollow [27] that STZ diabetic rats were less susceptible to acetaminophen-induced liver necrosis than normal rats. The authors suggested that the protective mechanism was related to an enhanced glucuronidation capacity in diabetic livers, resulting in the removal of toxic acetaminophen metabolites more rapidly than occurred in controls. The data were consistent with our findings and clearly indicated the significance of enhanced conjugation in the overall metabolism of foreign compounds.

In summary, our data demonstrate that glucuronidation is increased in hepatocytes of insulin-deficient rats and that the effect may be the result of altered carbohydrate metabolism in these cells. We postulate that such alterations increased availability of UDPGA in hepatocytes of diabetic rats; however, confirmation of this requires further study.

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Comparison of the *in vivo* effects of convulsant and optically active hypnotic barbiturates with their effects on the *in vitro* K^+-stimulated release of [^{3}H]acetylcholine*

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Barbiturates inhibit the release of acetylcholine (ACh) from the exposed cerebral cortex *in vivo* [1–3] and from the electrically stimulated, perfused superior cervical ganglion [4]. To examine inhibition of neurotransmitter release in the CNS *in vitro*, K^+-stimulated brain slice preparations have been used. Early studies showed that amobarbital and thiopental inhibit K^+-stimulated ACh and [^{14}C]ACh release from cerebral cortex slices [5, 6]. Later investigations indicated that this inhibition of K^+-stimulated ACh release occurs in other brain regions [7], is dose dependent and is a common characteristic of several members of the barbiturate drug class [8]. Further work using brain slices *in vitro* excluded the possibility that the effect on K^+-stimulated ACh release was a result of ACh synthesis inhibition by the barbiturates since there was no difference, as determined by a comparison of dose–response curves, between the phenobarbital inhibition of Ca^{2+}-dependent K^+-stimulated release of endogenous ACh and preformed radioactive ACh [9].

To test more rigorously the inhibition of K^+-stimulated ACh release in brain slices as a possible model system for the study of the mechanism of barbiturate depression of the CNS, we compared the *in vivo* effects of the stereoisomers of pentobarbital [*R*(+)-PB, *S*(−)-PB] and secobarbital [*R*(+)-SB, *S*(−)-SB] and the effects of the convulsant barbiturate 5-(2-cycohexylideneethyl)-5-ethyl barbituric acid (CHEB) [10] with their effects *in vitro* on K^+-stimulated [^{3}H]ACh release.

Materials and methods

PB and SB in their free acid forms were purchased from the Sigma Chemical Co., St. Louis, MO. The stereoisomers of these drugs were supplied through the offices of Dr. Robert E. Willette of NIDA. CHEB was provided by Dr. Hall Downes of the University of Oregon Health Sciences Center, Portland, OR.

Brain levels of barbiturates were determined in tissue from male ICR Swiss mice (25–35 g, Harlan Industries, Inc., Indianapolis, IN) that had been decapitated at loss of righting reflex after an intraperitoneal (i.p.) injection of racemic PB and SB or their stereoisomers and at convulsion after i.p. CHEB. The brains were removed, rinsed with saline, weighed, and stored in glass vials at −70° until time for assay. The barbiturates were extracted and measured spectrophotometrically by the method of Brodie *et al.* [11].

In those experiments in which inhibition of K^+-stimulated [^{3}H]ACh release was studied, the mice were decapitated and the brains were removed and dissected on ice. The forebrain region was obtained by making a vertical cut just caudal to the inferior colliculi. The tissue was cut into 0.4 mm slices by chopping in two directions at a 90° angle with a Brinkmann–McIlwain tissue chopper. The slices were suspended in 15 ml of low K^+ medium with 1 μCi/ml (0.1 μM) [^{3}H]choline (Amersham/Searle, Arlington Heights, IL, 10.1 Ci/mmole) and incubated for 10 min at 37° with gentle shaking. After this loading phase the slices were centrifuged at 4° and 19,000 *g* for 5 min. The resulting pellet was resuspended in 15 ml of low K^+ medium and 1-ml samples were placed in polyethylene tubes on ice. Following centrifugation as before, the pellets were resuspended in one of several different incubation media and incubated for 10 min at 37° with vigorous shaking. After this release phase the slices were again centrifuged as before. An 800-μl sample of the supernatant fluid was removed to assay for [^{3}H]ACh released into the medium. The remaining supernatant fluid was discarded and the pellet was homogenized in 1 ml of low K^+ medium. A 700-μl sample of the homogenate was taken to assay for [^{3}H]ACh remaining in the tissue. [^{3}H]ACh was measured in supernatant fluid and homogenate samples after extraction and purification as descibed previously [9, 12].

* A preliminary account of this work was presented at the meeting of the Federation of American Societies for Experimental Biology, Anaheim, CA, April 1980.