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STIMULATION OF *p*-NITROANISOLE *O*-DEMETHYLATION IN PERFUSED LIVERS BY XYLITOL AND SORBITOL*

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Abstract—Xylitol and sorbitol, two sugar alcohols which readily enter into pathways of hepatic carbohydrate metabolism, stimulated *p*-nitroanisole *O*-demethylation in perfused livers from fasted, but not fed, phenobarbital-treated rats. The increase in mixed-function oxidation correlated well with the production of NADH from the metabolism of xylitol and sorbitol (half-maximal stimulation for both processes was observed with concentrations between 0.1 and 0.2 mM). *p*-Nitroanisole metabolism by isolated hepatic microsomes was unaffected by the addition of xylitol and sorbitol; however, when NADH was added to microsomes, or was generated from sorbitol, sorbitol dehydrogenase and NAD^+, a synergistic increase in *p*-nitroanisole metabolism occurred. Ethanol (0.2 mM), which does not enter into pathways of carbohydrate metabolism, also caused an increase in the pyridine nucleotide redox state and stimululated *p*-nitroanisole *O*-demethylation in livers from fasted rats. In addition, sorbitol and xylitol stimulated *p*-nitrophenol conjugation in livers from fasted, phenobarbital-treated animals, probably by supplying substrate for increased UDP-glucuronic acid synthesis. The data indicate that sugars which influence the pyridine nucleotide redox state alter rates of mixed-function oxidation and conjugation in whole cells.

Nutritional intake has long been recognized to influence drug metabolism, and in large measure according to species and sex differences. For example, starvation generally decreases hepatic microsomal drug metabolism in male rats while it stimulates this activity in female rats and in both sexes of mice and rabbits (for review, see Ref. 1). Conversely, in rats fed a high carbohydrate diet, drug metabolism is decreased both *in vitro* and *in vivo* [1, 2]. Most studies exploring the influences of nutrition on drug metabolism have utilized relatively long-term dietary changes; much less information is available on the acute effects of carbohydrates in whole cell preparations. Thurman *et al.* reported that the infusion of glucose [3] or dihydroxyacetone [4] into perfused livers from fasted, phenobarbital-treated rats stimulated *p*-nitroanisole *O*-demethylase activity. Similarly, the addition of glucose or lactate to isolated hepatocytes from fasted, phenobarbital-treated rats increased alprenolol metabolism [5].

The conjugation of drug metabolites may also be influenced by the acute nutritional state. For example, rates of *p*-nitrophenol conjugation in perfused livers from phenobarbital-treated rats were greatest in fasted–refed animals, intermediate in animals which had free access to food, and least in 24-hr fasted rates [6]. These rates of conjugation correlated well with the hepatic glycogen content, rather than with glucuronyl transferase activity [6].

The mechanisms involved in the acute and chronic effects of nutrition on drug metabolism and conjugation remain obscure. These interactions are important, however, because fasting is often involved in certain types of chemotherapy, and because reduced food intake accompanies many pathological states. In addition, many precarcinogens undergo mixed-function oxidation before becoming metabolically active [7], and a relationship between the diet and cancer has been established in epidemiological studies [8].

Sorbitol and xylitol, two carbohydrates which are actively metabolized in hepatocytes, were employed in these studies to explore some of the acute interactions between carbohydrate metabolism and mixed-function oxidation in the perfused rat liver. The data indicate that sorbitol and xylitol increase the rate of *p*-nitroanisole *O*-demethylation in livers from fasted, but not well-fed rats. This stimulation most likely occurs by providing NADH for the mixed-function oxidase system. A preliminary account of this work has appeared elsewhere [9].

MATERIALS AND METHODS

Animals. Female Sprague–Dawley rats, 100–200 g, received sodium phenobarbital (1 mg/ml) in drinking water for 2 weeks, prior to perfusion experiments to induce the microsomal mixed-function oxidase enzymes [10]. Fasted animals were deprived of food for 24 hr prior to use.

Liver perfusion. Details of the perfusion technique have been described elsewhere. Livers were perfused with Krebs–Henseleit bicarbonate buffer, pH 7.4, saturated with an oxygen–carbon dioxide mixture (95:5) in a non-recirculating system. The fluid (37°) was pumped via a cannula placed in the vena cava

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past a Teflon-shielded oxygen electrode, before being discarded, in order to monitor continuously tissue viability. *p*-Nitroanisole (0.2 mM) was dissolved in Krebs–Henseleit bicarbonate buffer, and the continuous formation of *p*-nitrophenolate ion was monitored spectrally, as described previously [3]. Under these conditions, 4-nitrocatechol formation from *p*-nitrophenol was minimal (L. A. Reinke and R. G. Thurman, unpublished observation). Sorbitol and xylitol were dissolved in the buffer and infused into the perfusion fluid, entering the liver at final concentrations indicated in the text and figure legends.

Determination of glucuronide and sulfate conjugates of p-*nitrophenol in perfusate.* Where indicated, glucuronide and sulfate conjugates of *p*-nitrophenol were measured by adding 1.0 ml samples of perfusate to 0.5 ml of 0.1 M phosphate buffer, pH 7.4, which contained 275 units β-glucuronidase and 25 units sulfatase activities (Sigma. Chemical Co., St. Louis, MO). The samples were incubated for 3 hr at room temperature to hydrolyze all glucuronide and sulfate conjugates. The *p*-nitrophenol liberated was then measured spectrally at 436 nm.

Hepatic microsomal p-*nitroanisole* O-*demethylase activity.* Hepatic microsomes were prepared by standard techniques of differential centrifugation [11]. They were subsequently washed and resuspended in 0.15 M KCl. Assays were performed in 25 ml Erlenmeyer flasks containing 10 mM nicotinamide, 5 mM $MgCl_2$, 0.5 mM *p*-nitroanisole, microsomes (2–3 mg/ml) and an NADPH-generating system consisting of 0.4 mM $NADP^+$, 30 mM isocitrate dehydrogenase and 0.2 units isocitrate dehydrogenase (Sigma Chemical Co.), in a final incubation volume of 2.0 ml of 0.18 M phosphate buffer, pH 7.4. The incubations were initiated by the addition of the NADPH-generating system, and were terminated after 15 min by the addition of 0.5 ml of 0.6 M perchloric acid. The precipitated microsomal protein was removed by centrifugation, and 1.0 ml of the supernatant fraction was mixed with 0.1 ml of 12 N NaOH. 4-Nitrocatechol formed from *p*-nitrophenol represented approximately 20 per cent of the *p*-nitroanisole metabolized under these conditions. Its concentration was determined in the alkalinized supernatant fractions at 480 nm ($\varepsilon_{480} = 8.61 \cdot mM^{-1}$). The *p*-nitrophenol concentration was determined at 436 nm ($\varepsilon_{436} = 7.11 \cdot mM^{-1} \cdot cm^{-1}$) after correcting the absorbance for the contribution of 4-nitrocatechol ($\varepsilon_{436} = 3.85 \cdot mM^{-1} \cdot cm^{-1}$). Microsomal protein was determined by the biuret reaction [12], and *p*-nitroanisole *O*-demethylation activity was expressed as the sum of *p*-nitrophenol and 4-nitrocatechol formed $\cdot min^{-1} \cdot mg^{-1}$ of microsomal protein.

Surface fluorescence of pyridine nucleotides and flavoproteins. The redox state of tissues may be monitored noninvasively, employing surface fluorescence techniques. Certain oxidized flavoproteins fluoresce at 520 nm when excited at 460 nm, while reduced pyridine nucleotide fluorescence is excited at 366 nm and monitored at 450 nm. Changes in flavoprotein fluorescence reflect predominantly changes in the mitochondrial oxidation–reduction state, while pyridine nucleotide fluorescence indicates changes in both mitochondrial and extramitochondrial NAD^+ and $NADP^+$ coenzymes [13]. With this technique, the liver is illuminated alternately with pulses of 366 and 460 nm light. The emitted fluorescence is detected by the photomultiplier after passing through secondary filters having transmission maxima at 450 and 520 nm respectively.

The mechanical and electronic details of the double fluorometer are described elsewhere [14]. The apparatus consists of a high intensity xenon lamp, a photomultiplier, a combination of primary and secondary filters mounted on a rapidly (air-driven) rotating disc, and necessary electronic components.

RESULTS

The effects of xylitol and sorbitol on p-*nitroanisole* O-*demethylation in perfused livers.* The influence of

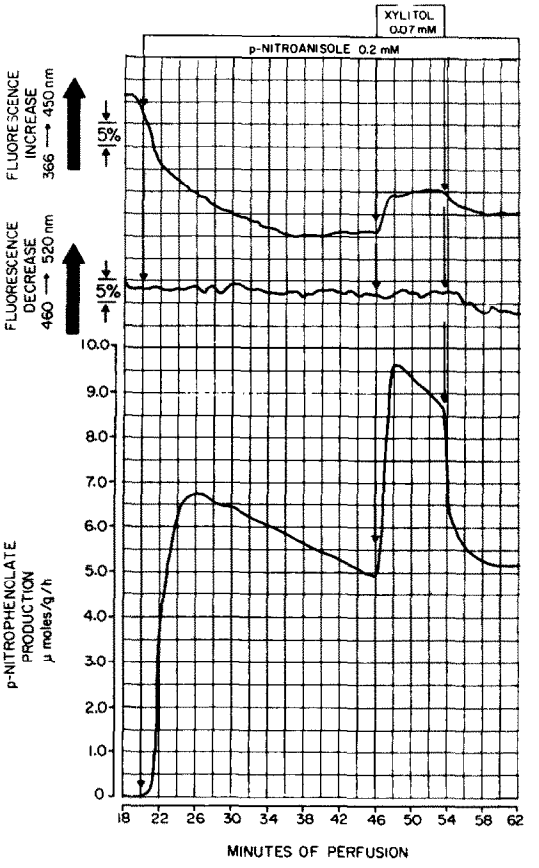

Fig. 1. Effect of xylitol on *p*-nitrophenolate production from *p*-nitroanisole and on pyridine nucleotide and flavoprotein fluorescence in a perfused liver from a fasted, phenobarbital-treated rat. The production of free *p*-nitrophenol was continuously assayed in the effluent perfusion fluid, as described in Materials and Methods. The introduction of *p*-nitroanisole (0.2 mM) and xylitol (0.07 mM) is designated by the horizontal bars and vertical arrows. Fluorescence increase (366 → 450 nm) represents reduction in pyridine nucleotide oxidation–reduction state, while fluorescence decrease (460 → 520 nm) represents flavoprotein reduction. One vertical scale division represents a 5 per cent change in fluorescence intensity.

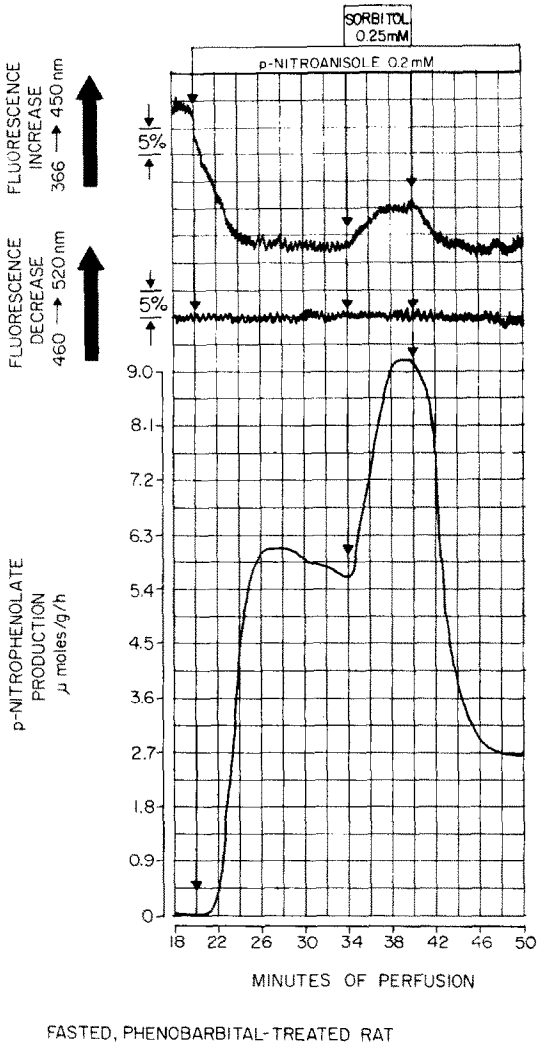

Fig. 2. Effect of sorbitol on *p*-nitrophenolate production from *p*-nitroanisole and on pyridine nucleotide fluorescence in a perfused liver from a fasted, phenobarbital-treated rat. The introduction of *p*-nitroanisole (0.2 mM) and of sorbitol (0.25 mM) is designated by the horizontal bars and vertical arrows. Other conditions are as in Fig. 1.

xylitol (0.07 mM) and of sorbitol (0.25 mM) on the production of free *p*-nitrophenol from *p*-nitroanisole in perfused livers from fasted, phenobarbital-treated rats is shown in Figs. 1 and 2 respectively. Both xylitol and sorbitol nearly doubled the rate of *p*-nitrophenol formation from *p*-nitroanisole (Figs. 1 and 2). The decrease in pyridine nucleotide fluorescence observed when *p*-nitroanisole infusion was initiated results from fluorescence quenching by *p*-nitroanisole and *p*-nitrophenol and, therefore, does not represent oxidation of these coenzymes [15]. The infusion of both sugar alcohols was accompanied by a fluorescence increase (reduction) in pyridine nucleotides, while flavoprotein fluorescence was unaffected (Figs. 1 and 2).

The effects of increasing concentrations of xylitol and sorbitol on free *p*-nitrophenol production from *p*-nitroanisole and on the pyridine nucleotide redox state in livers from fasted rats are shown in Fig. 3.

Both pyridine nucleotide fluorescence and *p*-nitrophenol formation increased in a step-wise fashion as the concentrations of the sugar alcohols were increased. Half-maximal changes in surface fluorescence and mixed-function oxidation occurred between 0.1 and 0.2 mM with both sorbitol and xylitol (Fig. 3).

Xylitol and sorbitol caused pyridine nucleotide reduction when infused into livers from fed, phenobarbital-treated rats (Fig. 4); however, the redox changes were smaller in this metabolic state than in fasted livers. Half-maximal pyridine nucleotide reduction was achieved with 0.5–0.8 mM carbohydrate (Fig. 4). In contrast to the large increases in *p*-nitrophenol formation (3–6 μmoles/g/hr) observed in livers from fasted rats (Figs. 1–3), only very small increases (less than 0.5 μmoles/g/hr) in free *p*-nitrophenol production from *p*-nitroanisole were observed in livers from fed rats (Fig. 4).

p-Nitrophenol is actively conjugated in the perfused rat liver, primarily via glucuronide and sulfate formation [16]. Therefore, samples of perfusate were incubated with glucuronidase and sulfatase to determine the influence of sorbitol and xylitol on the rates of total (free and conjugated) *p*-nitroanisole *O*-demethylation. In livers from fed, phenobarbital-treated rats, *p*-nitrophenol produced from *p*-nitroanisole is more actively conjugated (85 per cent) than

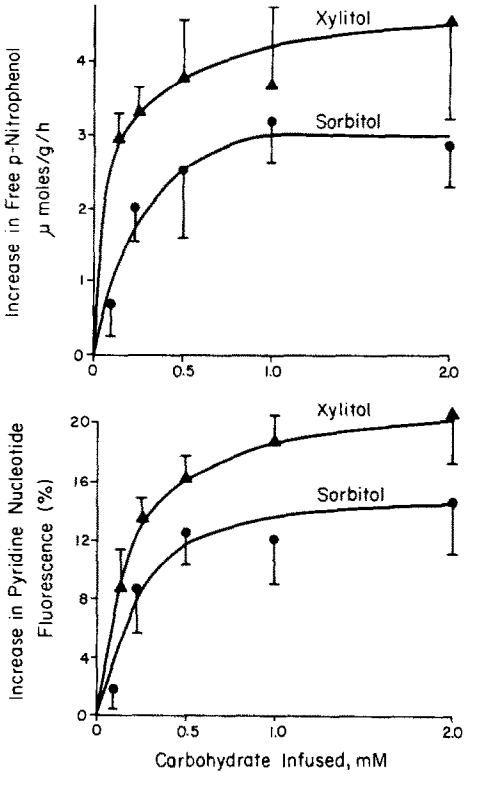

Fig. 3. Effects of xylitol and sorbitol on free *p*-nitrophenolate production and on pyridine nucleotide fluorescence in perfused livers from fasted, phenobarbital-treated rats. The data points for the various carbohydrate concentrations are means ± S.E.M. from four to six experiments, as illustrated in Figs. 1 and 2.

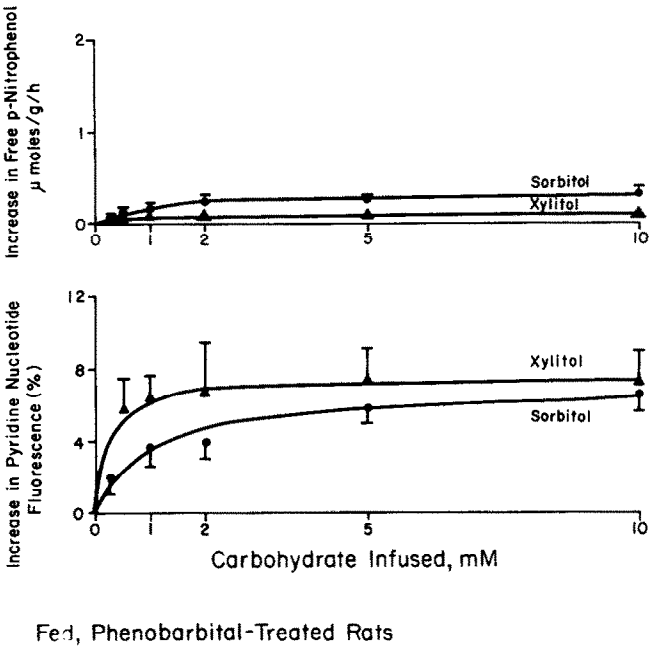

Fig. 4. Effects of xylitol and sorbitol on free *p*-nitrophenolate production and on pyridine nucleotide fluorescence in perfused livers from fed, phenobarbital-treated rats. Experiments such as those illustrated in Fig. 1 and 2 were performed in perfused livers from fed, phenobarbital-treated rats, utilizing various concentrations of xylitol and sorbitol. The data points are means ± S.E.M. from four to six experiments.

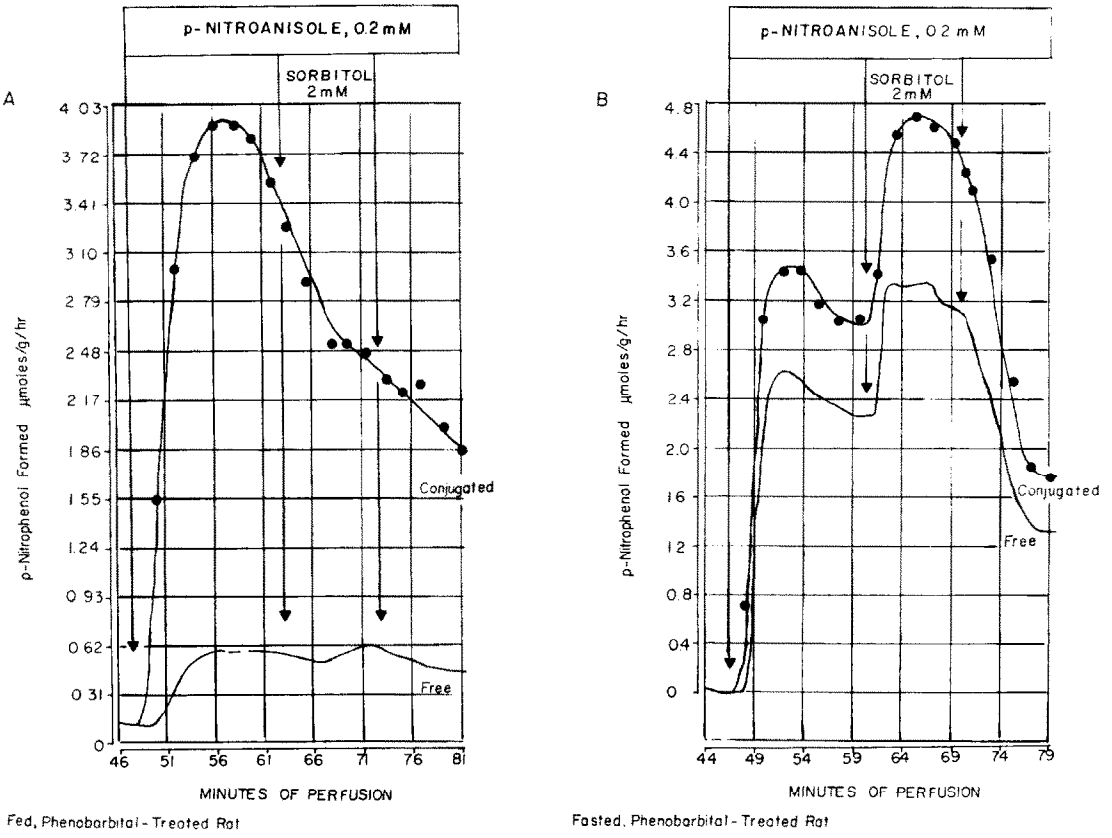

Fig. 5. Effects of sorbitol on free and conjugated *p*-nitrophenol in perfused livers from phenobarbital-treated rats. Samples of perfusate taken at various time points were treated as described in Materials and Methods for the determination of *p*-nitrophenol conjugates. The introduction of *p*-nitroanisole (0.2 mM) and of sorbitol (2.0 mM) is designated by the horizontal bars and vertical arrows. Panel A: fed rat. Panel B: fasted rat.

in livers from fasted rats (25 per cent; Fig. 5; see Ref. 6). In the fed state, the rate of conjugation subsequently declined as the rate of *p*-nitrophenol production from *p*-nitroanisole decreased (Fig. 5A). During sorbitol infusion, an increase in free *p*-nitrophenol formation was observed in livers from fasted animals (Fig. 5B). Sorbitol also caused the conjugation rate of *p*-nitrophenol in livers from fasted animals to increase slightly, from a rate of 0.84 ± 0.08 μmole/g/hr during the 10 min prior to sorbitol infusion to 1.36 ± 0.06 μmoles/g/hr in the presence of sorbitol (means ± S.D. for six determinations). In livers from fed rats, sorbitol infusion did not increase the total rate of *p*-nitrophenol formation from *p*-nitroanisole, although there was a reproducible tendency for free *p*-nitrophenol to increase slightly under these conditions (Fig. 5A). Xylitol infusion produced similar effects on *p*-nitrophenol formation and conjugation in livers from fed and fasted rats (not shown).

Ethanol increases the intracellular NADH redox state via the action of alcohol dehydrogenase [17]. The influence of ethanol (0.2 mM), on free and total *p*-nitrophenol formation from *p*-nitroanisole and on pyridine nucleotide and flavoprotein fluorescence is shown in Fig. 6. The infusion of ethanol caused a reduction in both pyridine nucleotide and flavoprotein redox states. In addition, both total production and unconjugated *p*-nitrophenol production were increased during ethanol metabolism (Fig. 6). Ethanol did not stimulate *p*-nitroanisole metabolism in livers from fed rats (not shown).

Effects of sorbitol and xylitol on microsomal p-nitroanisole O-demethylation. In isolated hepatic microsomes, sorbitol and xylitol had no effect on *p*-nitroanisole *O*-demethylation (Table 1). However, when the microsomes were fortified with NADH or with an NADH-generating system consisting of sorbitol, NAD^+, and sorbitol dehydrogenase, a stimulation of *p*-nitroanisole metabolism was observed. In the absence of NADPH, NADH supported only a low rate of mixed-function oxidation (Table 1). The effect of NADH + NADPH on *p*-nitroanisole *O*-demethylation was greater than the additive effect of either cofactor alone (i.e. NADH synergism [18, 19].

DISCUSSION

Stimulation of mixed-function oxidation by xylitol and sorbitol in perfused livers from fasted rats. In mammalian liver, xylitol is dehydrogenated, predominantly by an NAD^+-linked xylitol dehydrogenase, to form xylulose [20], and sorbitol is converted to fructose by an NAD^+-dependent sorbitol dehydrogenase [21]. Subsequently, both fructose and xylulose are phosphorylated and enter the glycolytic and gluconeogenic pathways at the triose phosphate level [20, 22]. In livers from fasted rats, sorbitol, xylitol and fructose are rapidly converted into glucose [23, 24]. It has been suggested that the metabolism of glucose via the pentose phosphate pathway is the predominant source of NADPH [25], the obligatory cofactor for mixed-function oxidation [26]. Livers from 24 hr-fasted animals contain less than 2% of the glycogen stores of well-fed animals [27] and, therefore, sorbitol and xylitol may stimulate mixed-function oxidation by providing substrate for NADPH generation through the pentose phosphate shunt. Indeed, the stimulation of drug metabolism by glucose in whole cells from fasted animals has been proposed to occur via this mechanism [3, 5]. Alternatively, the metabolism of glucose, sorbitol or xylitol could indirectly increase cytosolic NADPH by supplying pyruvate for the malic enzyme shuttle mechanism [3, 5].

The pyridine nucleotide reduction produced by sorbitol and xylitol correlated with the increase in *p*-nitrophenol formation from *p*-nitroanisole, with half-maximal changes of both phenomena occuring

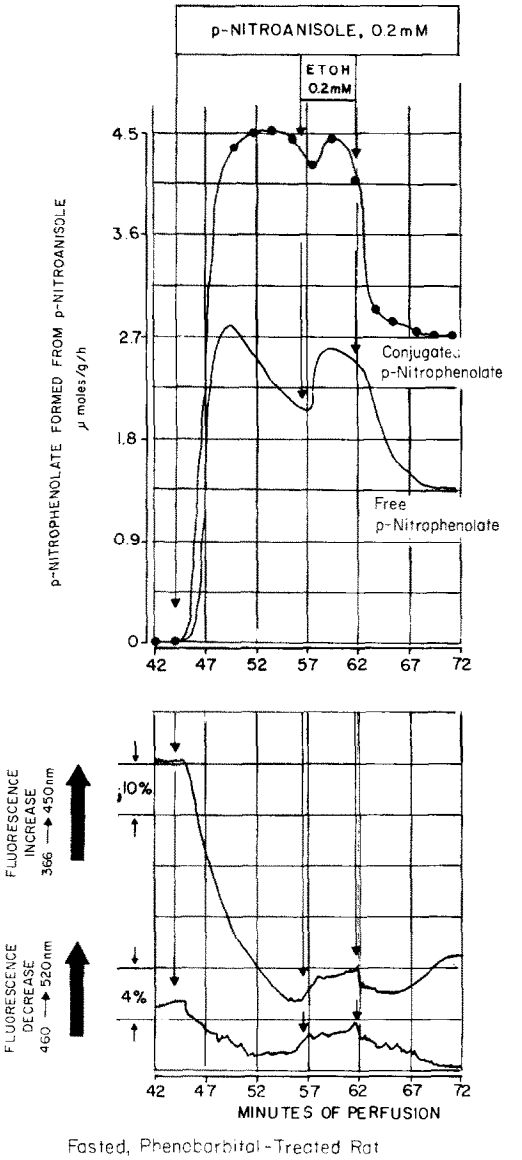

Fig. 6. Effect of ethanol (0.2 mM) on *p*-nitroanisole *O*-demethylation and pyridine nucleotide and flavoprotein fluorescence in a perfused liver from a fasted, phenobarbital-treated rat. Conjugates of *p*-nitrophenol were determined as described in Materials and Methods. The introduction of *p*-nitroanisole (0.2 mM) and of ethanol (0.2 mM) is designated by the horizontal bars and vertical arrows. Other conditions are as in Fig. 1.

Table 1. Effects of sorbitol and xylitol on hepatic microsomal *p*-nitroanisole *O*-demethylation

Addition	*p*-Nitroanisole *O*-demethylation activity* (nmoles/min/mg microsomal protein)
None	1.20 ± 0.06
Xylitol (10 mM)	1.12 ± 0.01
Sorbitol (10 mM)	1.13 ± 0.01
Sorbitol (10 mM), NAD^+ (1.0 mM), Sorbitol dehydrogenase (0.5 units)	1.61 ± 0.01†
NADH (1.0 mM)	1.62 ± 0.05†
NADH (1.0 mM) (no NADPH)	0.27 ± 0.05†

* Values are means ± S.D. of triplicate determinations, as described in Materials and Methods, in hepatic microsomes from fasted, phenobarbital-treated rats. NADH and sorbitol dehydrogenase (Sigma Chemical Co.) were added to the flasks just prior to the NADPH-generating system in their respective assays.

† Significantly different ($P < 0.001$) with respect to flasks with no additions.

with 0.1–0.2 mM carbohydrate (Fig. 3). The stimulation of *p*-nitroanisole *O*-demethylase activity in hepatic microsomes when incubated with an NADH-generating sorbitol-metabolizing system or NADH alone (Table 1) simulates the effect of sorbitol in the perfused liver (Fig. 1) and suggests identical mechanisms. NADH synergism of NADPH-dependent drug metabolism is well established [18, 19]. The mechanism involved in this synergism is not well understood, but probably involves donation of electrons to cytochrome P-450 via cytochrome b_5 [18, 19]. NADH synergism in microsomes is also apparent in these studies, since NADH addition or generation resulted in a greater increase in *p*-nitroanisole *O*-demethylation activity than was observed from summing the effect of NADH and NADPH alone (Table 1). Thus, NADH synergism of mixed-function oxidation is also a possible explanation of the stimulatory effect of xylitol and sorbitol.

Stimulation of *p*-nitroanisole *O*-demethylation by low concentrations of ethanol in livers from fasted rats appears to result from NADH synergism of mixed-function oxidation [9]. This stimulation of *p*-nitrophenol formation could be blocked by the inhibitor of alcohol dehydrogenase, 4-methylpyrazole [28]. was insensitive to amino-oxyacetate, a transaminase inhibitor [29], and correlated well with NADH produced from ethanol metabolism. Ethanol is not a carbon source for gluconeogenesis in the perfused liver, since it is metabolized to acetaldehyde and then to acetate [30]. In addition, ethanol inhibits gluconeogenesis [31]. Thus, ethanol (0.2 mM) stimulation of mixed-function oxidation in livers from fasted animals must occur through NADH production without producing carbohydrate intermediates. Production of NADH directly from the metabolism of xylitol and sorbitol rather than via carbohydrate intermediates is most likely the predominant factor in the stimulation of *p*-nitroanisole *O*-demethylation by these sugar alcohols. This possibility, however, does not exclude other mechanisms, e.g. activation of pentose phosphate shunt activity.

Sorbitol and xylitol do not stimulate mixed-function oxidation of *p*-nitroanisole in livers from fed rats (Fig. 5A) where large glycogen stores are present [27]. Furthermore, sufficient NADH may be produced from normal glycolytic flux in this metabolic state to saturate the cytochrome b_5 system. In this metabolic state, neither the production of more NADH from the metabolism of sorbitol or xylitol, nor the addition of excess carbohydrate would be expected to stimulate mixed-function oxidation, in agreement with the experimental observation (Fig. 5A).

Interactions of sorbitol and xylitol with conjugation of p-nitrophenol in perfused livers. *p*-Nitrophenol is more actively conjugated in livers from fed, phenobarbital-treated rats than in livers from fasted animals [6], (Fig. 5). Differences in rates of conjugation were more closely related to carbohydrate reserves, as indicated by glycogen levels, than to activities of UDP-glucuronyl transferase [6]. In livers from fasted, phenobarbital-treated rats, sorbitol increased not only the rate of mixed-function oxidation, but also the rate of conjugation of *p*-nitrophenol (Fig. 5, Results). While the stimulation of *p*-nitroanisole *O*-demethylation by sorbitol may be largely explained by NADH synergism (see above), the most likely explanation for the increased rate of conjugation is increased UDP-glucuronic acid synthesis via gluconeogenesis from sorbitol

Conversely, in livers from fed rats the infusion of sorbitol or xylitol tended to increase the concentration of free *p*-nitrophenol in the perfusate (Figs. 4 and 5). This is due most likely to inhibition of conjugation of *p*-nitrophenol (Fig. 5). Sorbitol has also been shown to inhibit glucuronidation in isolated hepatocytes, probably via redox inhibition of UDP-glucose dehydrogenase [32].

In conclusion, these studies demonstrate that hepatic drug metabolism and disposition can be altered acutely by the carbohydrate supply to the liver. Removal of glycogen stores through fasting markedly decreased the conjugation of *p*-nitrophenol and established conditions where NADH generated from the metabolism of xylitol and sorbitol stimulated mixed-function oxidation. Additionally, infusion of these carbohydrates increased the rates of conjugation in livers from fasted animals. These findings may be relevant to the disposition of chemotherapeutic agents in pathological states where

reduced food intake and/or intravenous carbohydrate infusion are commonly employed.

Note added in proof—In livers from fasted, phenobarbital-treated rats, sorbitol (5 mM) infusion increased measured NADH by 250 per cent, confirming the increase in pyridine nucleotide fluorescence observed (Fig. 3).

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