Control of the Removal of Reducing Equivalents from the Cytosol in Perfused Rat Liver*  

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SUMMARY  

The rat liver perfusion previously employed was adapted to a flow-through system in which samples of effluent perfusion medium were collected for measurement of metabolic products at 2-min intervals by means of a fraction collector. Overnight fasted rats were used to avoid complications of gluconeogenesis from endogenous glycogen. Since xylitol conversion to glucose and lactate by the liver is associated with the production of NADH in the cytosol, the rates of production of these metabolic end products can be used to provide a measure of the rate of NADH production and utilization.  

Xylitol metabolism was predominantly cyanide sensitive showing an obligatory involvement of mitochondrial respiration for removal of reducing equivalents from the cytosol. The participation of various shuttles for the transport of reducing equivalents into the mitochondria was investigated by infusion of the oxidized or reduced partners of cytosolic or mitochondrial NAD-linked dehydrogenases (e.g., ethanol, β-hydroxybutyrate), or specific inhibitors such as rotenone, amobarbital, cyanide, and the transaminase inhibitor aminooxyacetate. The influence of flux in the mitochondrial electron transport chain on the rate of removal of NADH from the cytosol was studied by infusion of 2,4-dinitrophenol to provide an energy drain on mitochondrial ATP and artificial electron acceptors to bypass rate-limiting electron transport steps.  

The studies show that xylitol metabolism was not controlled by the activity of NAD-xylitol dehydrogenase but by the rate of reoxidation of NADH. This process was regulated by the rate of transfer of reducing equivalents into the mitochondria and by the rate of electron flux to oxygen. Evidence was obtained for the participation of both NAD- and flavin-linked shuttles in the over-all transport of reducing equivalents from cytosol to mitochondria. The flavin-linked shuttle may be identified with the α-glycerophosphate shuttle which operates by virtue of the abnormally high cytosolic α-glycerophosphate concentration produced during xylitol metabolism. Involvement of the NAD-linked malate-aspartate shuttle was shown by the sensitivity of glucose formation from xylitol to aminooxyacetate and β-hydroxybutyrate. The relevance of these findings to the control of ethanol utilization by the liver is discussed.  

In the previous paper it was shown that xylitol is rapidly metabolized in the perfused rat liver by a cytosolic NAD-linked xylitol dehydrogenase, and that glucose is the main carbon end product (1). Competition between dehydrogenases was illustrated by the use of [14C]lactate added to the perfusion fluid separately or in combination with xylitol. The results showed that glucose formation from lactate was inhibited much more than glucose formation from xylitol when both substrates were present together. Glucose formation from D-xylulose, the product of NAD-xylitol dehydrogenase, involves phosphorylation of the pentose by ATP to D-xylulose-5-P which is converted to fructose-6-P and triose phosphates by the transaldolase and transketolase reactions of the pentose phosphate cycle (2). Thus, unlike lactate gluconeogenesis, xylitol gluconeogenesis is associated with the net generation of NADH in the cytosol, which is equal to 1.2 times the rate of glucose production. Reoxidation of cytosolic NADH, therefore, is an obligatory step for xylitol metabolism, and its rate can be conveniently monitored by measuring the rate of glucose production by livers perfused with xylitol as substrate (3).  

Data described in this paper illustrate the use of a flow-through liver perfusion system for quantitative investigations of the control of cytosolic-mitochondrial interactions mediated by NAD- and flavin-linked carbon shuttles. The results obtained by the use of xylitol as a donor of reducing equivalents to NAD in the cytosol provide basic information on the control of intracellular hydrogen transport processes which is of relevance to the problem of ethanol utilization by the liver (4).  

EXPERIMENTAL PROCEDURE  

Animals—Male albino rats of Holtzman strain, 180 to 200 g in weight, were used. Food was withheld from the animals 24 to 30 hours prior to the perfusion experiments.
**Perfusion Technique**—In general, the experimental methods were similar to those used in the previous paper (1). However, the liver perfusion was adapted to a flow-through system in order to follow variations in the rate of glucose formation more conveniently than in the normal recirculating perfusion system. Because of the large volumes of fluid required, albumin was omitted from the perfusion medium. Krebs bicarbonate buffer, containing half-calcium concentration to compensate for omission of albumin, was used as the basic perfusion fluid. The lowering of oncotic pressure by the absence of protein or other high molecular weight substitute appeared to have little deleterious effect on liver function for perfusion periods up to 90 min. Thus, liver swelling was no greater than in the presence of albumin, oxygen consumption was not affected, and intracellular K+ was well retained.

The perfusion fluid was contained in a 2-liter volumetric flask immersed in a water bath maintained at 37°C, and was saturated with a gas mixture containing 95% O₂ and 5% CO₂. Fluid was pumped from this reservoir at a rate of 25 to 30 ml per min into a rotating disc oxygenator (5), which ensured saturation of the fluid with the oxygen gas mixture, and was pumped out at the same rate through the liver via a cannula placed in the portal vein. Fluid left the liver via a cannula placed in the superior vena cava, and passed immediately through a 0.2-ml chamber containing a micro Clark oxygen electrode to a stream-splitting device which allowed part of the fluid to run to waste while the remainder (approximately 2 ml per min) was collected at 2-min intervals with a time-programmed fraction collector. Substrates and inhibitors were continuously added by infusion pumps to the fluid immediately prior to the liver to give the calculated arterial concentrations indicated in the figure legends.

**Analytical Techniques**—Glucose in the collected effluent fractions was assayed enzymatically by the glucose-6-P dehydrogenase-hexokinase method (6). Automatic pipetting and sampling devices attached to a spectrophotometer fitted with a flow cell (Gilford) permitted the assays to be completed with a lag of only a few minutes. Lactate was assayed similarly by means of lactate dehydrogenase, with the reaction complete within 5 min.

Fluoroscence measurements from the surface of the liver and absorption measurements through a liver lobe were monitored as previously described (1, 7, 8).

**Expression of Results**—Data obtained with individual livers are given in the figures in order to increase their clarity. However, each experiment was repeated four or five times with variations, and representative examples were chosen for presentation.

**RESULTS**

**Effect of Substrate Oxidants and Reductants on Glucose Formation from Xylitol and Lactate**—The rate of glucose production by livers from fasted rats perfused with the flow-through method in the absence of added substrate was 5 to 10 μmoles/100 g rat weight per hour. Fig. 1 shows that infusion of 5 mM xylitol produced a gradual increase in the rate of glucose production over a 15- to 20-min time interval to a maximum of about 90 μmoles/100 g rat weight per hour. In general, the rate of glucose production by livers perfused with the flow-through method was somewhat lower than those obtained with the recirculation system (1), but control experiments showed that it could be maintained for at least 60 min. Infusion of 5 mM acetoacetate over the time interval denoted by the horizontal bar in Fig. 1 caused an immediate slight stimulation of glucose production, which was not sustained. On the other hand, infusion of 10 mM β-hydroxybutyrate produced a prompt 25 to 30% inhibition. This inhibition was relieved, and glucose formation stimulated above control levels by infusion of 25 μM artificial electron acceptor tetramethyl-p-phenylenediamine.

The reproducibility of the xylitol stimulation of glucose production and inhibition by β-hydroxybutyrate is illustrated in Fig. 2, which also shows the rapid stimulation of glucose formation produced by 5 μM phenazine methosulfate. Methylene blue (20 μM) produced similar effects. This stimulation of glucose formation by artificial electron acceptors was cyanide sensitive.

The rate of oxygen consumption by livers respiring on endogenous substrates in the flow-through liver perfusion system was in the range of 500 to 600 μatoms/100 g rat weight per hour. 

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**Fig. 1. Effects of acetoacetate (5 mM), β,β-hydroxybutyrate (10 mM) and tetramethyl-p-phenylenediamine (TMPD) (25 μM) infusion on glucose production from xylitol in perfused rat liver.**

**Fig. 2. Effects of β,β-hydroxybutyrate (10 mM) and phenazine methosulfate (PMS) (5 μM) infusion on glucose production from xylitol in perfused rat liver.**
The effect of xylitol infusion on respiration was small and variable, but β-hydroxybutyrate infusion invariably increased respiration by 30 to 40 μatoms of oxygen per 100 g rat weight per hour. The artificial electron acceptors, on the other hand, stimulated oxygen consumption to more than 1000 μatoms/100 g rat weight per hour.

The above results show that when reducing equivalents are generated directly in the mitochondria by addition of the reduced partner, namely β-hydroxybutyrate, of a mitochondrial NAD-linked dehydrogenase, the rate of reoxidation of NADH generated from xylitol dehydrogenase is diminished. Fig. 3 shows that infusion of 2 mM ethanol, a reducing agent of the cytosolic NAD pool, also produced a substantial inhibition of glucose formation from xylitol. The figure also illustrates the rapid reversibility of the β-hydroxybutyrate inhibition once infusion was stopped. In this experiment, respiration of the liver decreased by 50 μatoms/100 g rat weight per hour when infusion of β-hydroxybutyrate was stopped, but ethanol infusion had a negligible effect on the oxygen consumption. Other experiments showed that infusion of 1 mM acetaldehyde in the presence of xylitol produced a stimulation of glucose formation and an inhibition of lactate formation, indicating that acetaldehyde acted more as an NADH acceptor with alcohol dehydrogenase than as an NADH donor via acetaldehyde dehydrogenase. However, in the presence of 1 mM pyrazole to inhibit alcohol dehydrogenase (9), an inhibition of glucose formation from xylitol was observed, suggesting that under these conditions it functioned as a donor of reducing equivalents.

Ethanol infusion also inhibited glucose formation from lactate in the flow-through perfusion system. Fig. 4A shows that when 2 mM ethanol was infused together with 1.5 mM L(+)-lactate, the rate of gluconeogenesis was about 55 μmoles/100 g rat weight per hour. Upon stopping ethanol infusion, glucose production increased to 80 to 90 μmoles/lOO g rat weight per hour. In Fig. 4B, it is seen that infusion of 4 mM β-hydroxybutyrate stimulated gluconeogenesis from lactate, contrary to its inhibitory effect on glucose formation from xylitol. Shown also in Fig. 4B is the inhibitory effect of ethanol on lactate gluconeogenesis. Clearly, with lactate as substrate, an increased rate of generation of reducing equivalents in the mitochondria via β-hydroxybutyrate dehydrogenase affects gluconeogenesis by a different mechanism than when reducing equivalents are generated directly in the cytosol via alcohol dehydrogenase.

Effect of Increased Electron Transport on Glucose Production from Xylitol—Fig. 5 shows the effect of increasing concentrations of 2,4-dinitrophenol to a liver perfused with 10 mM xylitol. Dinitrophenol concentrations of 5 and 20 μM produced successive increases of oxygen consumption and glucose production. With 100 μM dinitrophenol, although respiration was further increased, glucose production became inhibited. However, it was restored to control levels when the dinitrophenol infusion was terminated. This experiment shows that when flux through the mitochondrial electron transport chain was increased due to partial uncoupling of oxidative phosphorylation, flux in the pathway from xylitol to glucose increased. However, since ATP is required for the phosphorylation of D-xylulose, severe impairment of mitochondrial ATP production is associated with an inhibition of xylitol conversion to glucose.

Flux through the phosphorylating electron transport chain can also be increased by increasing the rate of mitochondrial ATP utilization for biosynthetic reactions. As shown by Hems et al. (10), this may be achieved in the perfused rat liver by addition of ornithine and NH₄Cl, which induces a stimulation of oxygen consumption and urea formation. Fig. 6 shows that infusion of 2 mM ornithine together with 10 mM NH₄Cl, after prior infusion of xylitol, produced a 40% stimulation of glucose production. Ornithine alone had a negligible effect on glucose formation. Oxygen consumption increased from 635 to 717 μatoms/100 g rat weight per hour. Subsequent infusion of 10 mM β-hydroxybutyrate decreased glucose production from 120 to 55 μmoles/100 g rat weight per hour although the oxygen consumption of the liver increased by 150 μatoms/100 g rat weight per hour. Infusion of 25 μM methylene blue caused a prompt restoration of glucose formation.

Fig. 3. Effects of D,L-β-hydroxybutyrate (10 mM) and ethanol (2 mM) infusion on glucose production from xylitol in perfused rat liver.

Fig. 4. Top, effects of ethanol (2 mM) infusion on glucose production from L(+)-lactate (1.5 mM) in perfused rat liver. Bottom, effects of D,L-β-hydroxybutyrate (4 mM) and ethanol (2 mM) on glucose production from L(+)-lactate (1.5 mM).
Oxygen Consumption

Minutes Of Perfusion

Glucose Production

Minutes Of Perfusion

FIG. 5. Effects of 2,4-dinitrophenol infusion on oxygen consumption and glucose production from xylitol in perfused rat liver.

Pathways Involved in Transport of Reducing Equivalents from Cytosol to Mitochondria—In order to assess the relative significance of the malate-aspartate and α-glycerophosphate shuttles for the transport of NADH into the mitochondria, the transaminase inhibitor aminooxycacetate (11) was used to inhibit flux through the malate-aspartate cycle (12) in livers perfused with xylitol. Fig. 7 shows that 0.5 μM aminooxycacetate initially produced a 20% inhibition of glucose production but that after about 10 min the inhibition was completely reversed. Lactate production increased from 5 to 15 μmoles/100 g rat weight per hour after xylitol infusion and was not appreciably affected by aminooxycacetate. Simultaneous infusion of 4 mM β-hydroxybutyrate together with xylitol and aminooxycacetate produced a partial inhibition of glucose formation and an almost complete inhibition of lactate formation, while 25 μM methylene blue reversed these changes. Oxygen consumption decreased initially by 40 μatoms/100 g rat weight per hour after aminooxycacetate addition and increased by about the same amount after β-hydroxybutyrate infusion. The inhibitions of lactate and glucose formation from xylitol in the presence of aminooxycacetate and β-hydroxybutyrate indicate that the mechanism responsible for the transfer of reducing equivalents across the mitochondrial membrane is still sensitive to competition with mitochondrial NAD-linked dehydrogenases.

The effectiveness of aminooxycacetate as an inhibitor of the malate-aspartate shuttle in livers perfused with xylitol was investigated further with the aid of the surface fluorometry technique for measurement of flavin and pyridine nucleotide oxidation-reduction changes (7). These measurements were made on livers perfused with recirculation of the perfusion fluid rather than with the flow-through system. Fig. 8A shows the responses obtained with normal livers, while Fig. 8B shows results obtained in the presence of aminooxycacetate. Besides flavin and pyridine nucleotide oxidation-reduction changes, the absorption change at 560 nm (575 nm as reference), attributable to cytochrome b was also recorded. In Fig. 8A, an anoxic cycle is shown first for internal calibration of the traces. Addition of xylitol after 60 min of perfusion resulted in a partial reduction of both flavin and pyridine nucleotides, and a small but definite increase of cytochrome b absorption. Subsequent addition of ethanol caused further, but still incomplete, reductions of the flavin and pyridine nucleotide components. Fig. 8B shows that addition of xylitol and ethanol in the presence of aminooxycacetate caused successive further reduction of pyridine nucleo-
Fig. 8. Effects of xylitol and ethanol on flavin and pyridine nucleotide fluorescence and cytochrome b absorption of rat livers perfused in the absence (A) and presence (B) of 0.2 mM aminooxyacetate.

tides, but had no demonstrable effect on flavin fluorescence or cytochrome b absorption.

Although increased cytochrome b₄ absorption may contribute to the total absorption changes during the anoxic cycle, it is unlikely to interfere with cytochrome b oxidation-reduction changes in the aerobic state. Consequently, the increased absorption in the 560-nm region observed after xylitol addition may be interpreted as the response of cytochrome b to an increased steady state level of reduction of mitochondrial flavin and pyridine nucleotide pools. This observation lends support to the previous conclusion that flavin fluorescence changes in the perfused liver are mainly of mitochondrial origin and reflect principally oxidation-reduction changes of lipoic dehydrogenase (7). The pyridine nucleotide fluorescence changes observed after xylitol and ethanol additions to control livers indicate an elevated state of reduction of both cytosolic and mitochondrial NAD pools. Since lipoic dehydrogenase is considered to be in approximate equilibrium with the mitochondrial NAD pool (13, 14), the increased level of reduction of the pyridine nucleotides observed after xylitol and ethanol additions to control livers indicate an elevated state of reduction of both cytosolic and mitochondrial NAD pools. The conclusion is reached, therefore, that in the perfused liver aminooxyacetate is effective in inhibiting the transfer of reducing equivalents from the cytosolic to the mitochondrial space via the malate-aspartate shuttle. The data also indicate that other possible NAD-linked hydrogen transport shuttles are probably relatively inactive under the conditions of the experiment.

Transport of reducing equivalents into the mitochondria by means of the malate-aspartate and α-glycerophosphate shuttles can also be distinguished by the use of rotenone or amobarbital which inhibit mitochondrial electron transport between NADH and cytochrome b. α-Glycerophosphate donates electrons to a flavoprotein pool situated on the oxygen side of the site of inhibition, so that the α-glycerophosphate shuttle should be insensitive to inhibition by rotenone or amobarbital. Fig. 9 shows the effect of xylitol addition after rotenone on the fluorescence responses of flavin and pyridine nucleotides, and on the oxygen tension of fluid leaving the perfused rat liver. The numbers on the effluent oxygen trace refer to oxygen consumption rates in microatoms per 100 g rat weight per hour. The liver was depleted of endogenous substrates by prior perfusion for 1 hour. Addition of rotenone caused a partial reduction of the flavin and pyridine nucleotides, these responses being largely mitochondrial (1). Oxygen uptake by the liver was inhibited from 835 to 546 μatoms/100 g rat weight per hour. The subsequent addition of xylitol produced a small further increase of the flavin reduction state, and a large increase in the state of reduction of the pyridine nucleotides as the cytosolic NAD systems became reduced with addition of substrate. The oxygen consumption of the liver oxygen consumption of the liver increased by 50 to 60 μatoms/100 g rat weight per hour after a short delay, indicating increased flux through the α-glycerophosphate shuttle. Thus, xylitol provides the carbon substrate required to build up the intracellular α-glycerophosphate pool, and produces increased electron flux from the high potential flavin pool (14, 15) to oxygen. A second cycle of anoxia shows that both the flavin and pyridine nucleotide pools were incompletely reduced by the combination of rotenone and xylitol. Antimycin A and cyanide caused further successive increases in the state of reduction of both flavin and pyridine nucleotides along with successive inhibitions of the oxygen consumption.
The data presented in Fig. 9 indicate that xylitol metabolism was active in the presence of rotenone due to removal of reducing equivalents from the cytosol by means of a flavin-linked transport mechanism, which may be identified as the α-glycerophosphate shuttle. Fig. 10 shows measurements of the rates of glucose and lactate production from xylitol in a liver perfused with the throw-through system after successive additions of amobarbital (amytal) and cyanide. Other experiments showed that similar results were obtained with rotenone instead of amobarbital, and antimycin A instead of cyanide. Addition of amobarbital produced a rapid fall in the rate of glucose production from 86 to 40 μmoles/100 g rat weight per hour and a rise of lactate production from 16 to 55 μmoles/100 g rat weight per hour. Since amobarbital inhibits the reoxidation of mitochondrial NADH, oxidation of pyruvate is prevented, so that glucose and lactate become the only end products of xylitol metabolism. The rate of NADH production from xylitol metabolism thus becomes equal to the rate of xylitol uptake, which may be calculated from the sum obtained by multiplying the rate of glucose production by 1.2 and the rate of lactate production by 0.6. These data are plotted as solid triangles in Fig. 10. Conversion of xylitol to pyruvate and subsequent oxidation of pyruvate in the citric acid cycle in livers perfused with xylitol alone may initially account for 50% of the xylitol uptake, as previously shown (1). In this experiment, the calculated xylitol uptake uncorrected for pyruvate oxidation prior to addition of amobarbital was 100 μmoles/100 g rat weight, per hour, and decreased to 80 μmoles/100 g rat weight per hour after amobarbital addition. Thus, the inhibitor certainly produced some decrease of xylitol metabolism although the exact amount cannot be calculated from the available data. Diminished glucose and increased lactate production can be accounted for by increased phosphofructokinase activity, since amobarbital is known to increase ADP and AMP levels in the liver (16). After inhibition of the mitochondrial electron transport chain by addition of cyanide, glucose production fell rapidly to about 5 μmoles/100 g rat weight per hour, while lactate production initially increased (probably due to additional adenine nucleotide activation of phosphofructokinase), and subsequently decreased as the capacity of the liver to reoxidize NADH diminished. The calculated xylitol uptake fell to 40 μmoles/100 g rat weight per hour after cyanide addition, and decreased further by 75% upon disoxygenation of the liver. The small residual rate of lactate formation of 30 μmoles/100 g rat weight per hour obtained with anoxic perfusion probably represents a small oxygen leak from the surface of the liver or breakdown of residual glycogen. From this data we may conclude that the maximum rate of NADH reoxidation by extramitochondrial pathways is about 30 μmoles/100 g rat weight per hour, and that flux through the α-glycerophosphate shuttle is about 40 μmoles/100 g rat weight per hour.

Loss of carbon from xylitol by oxidation of pyruvate in the citric acid cycle can also be prevented by arsenite inhibition of lipoic dehydrogenase of the pyruvate dehydrogenase complex. Fig. 11 shows that the response of the liver to 0.2 mM arsenite infusion with xylitol as substrate was similar to that obtained after amobarbital inhibition, namely a decrease of glucose formation after an initial stimulation, and an increase of lactate.
formation. Since the oxygen consumption of the liver decreased by 30% during arsenite infusion, it may be presumed that decreased ATP production by the citric acid cycle resulted in an activation of phosphofructokinase, and hence an increased flux through the glycolytic pathway to lactate. The calculated rate of xylitol uptake diminished to about 80 μmoles/100 g rat weight per hour and was further decreased by 60% after cyanide addition. Subsequent addition of 25 μM phenazine methosulfate as an artificial electron acceptor increased the rate of lactate formation and produced a small stimulation of oxygen consumption (from 270 to 380 μatoms/100 g rat weight per hour) indicating an increased rate of NADH reoxidation in the cytosol, presumably via the cytochrome P-450 and cytochrome b₄ electron transport pathways (17).

**DISCUSSION**

Considerable confusion exists in the literature concerning the nature and control of the physiological pathways responsible for the transfer of reducing equivalents from the cytosol to the mitochondria in the intact cell (18). Largely on the basis of enzyme distribution patterns in the cytosol and mitochondria of various tissues and the known impermeability of the mitochondrial membrane to NADH, Bücher and Klingenberg (19) postulated an indirect transfer of reducing equivalents into mitochondria via α-glycerophosphate and malate. In a critical evaluation of a number of substrate oxidation-reduction cycles, Borst (12) concluded that the α-glycerophosphate cycle could account for all the glycolytic NADH oxidation in flight muscle, for part of it in rat liver, but for relatively little in rat heart. The role of the α-glycerophosphate shuttle for transport of extramitochondrial NADH to the respiratory chain is now well established for insect muscle (20, 21), some tumor tissues (22, 23), and yeast (24). The original postulate of a simple malate-oxalacetate exchange across the mitochondrial membrane was amended by Borst (25) on the grounds of the poor permeability of the mitochondrial membrane to oxalacetate at physiological concentrations. More recent observations have confirmed this point, although it is clear that oxalacetate transport can occur at nonphysiological concentrations (26, 27). Borst proposed an intramitochondrial transamination of oxalacetate with glutamate to form aspartate and α-ketoglutarate, followed by transport of these products to the cytosol for transamination back to glutamate and oxalacetate. The malate-aspartate cycle, therefore, involves an influx of malate and glutamate and efflux of α-ketoglutarate and aspartate from the mitochondria.

Evidence for the existence of specific dicarboxylic anion transport mechanisms across the membrane of most mammalian mitochondria has been obtained from a number of laboratories (26, 32). A possible difficulty with this type of NAD-linked shuttle can result only in an equilibrium of the oxidation-reduction potential on both sides of the membrane without appreciable transport. On the basis of measurements of the substrate partners of NAD-linked dehydrogenases located in the cytosolic and mitochondrial spaces, which may be used to calculate the oxidation-reduction potential of free NAD (19, 33), it is generally accepted that the mitochondrial NAD oxidation-reduction potential under a wide variety of metabolic conditions is considerably more negative than that of the cytosol. This has been demonstrated, too, in perfused rat liver under conditions of ethanol (34) or xylitol (1) metabolism, when NADH transport into mitochondria is high. Transport of reducing equivalents into mitochondria, therefore, appears to be against a concentration gradient when mediated via NAD linked shuttles, and should be energy linked. So far no definitive measurements have been made of the stoichiometry of the energy requirements for the malate-aspartate shuttle, although an energy dependency has been illustrated in the sense...
that the rate of NADH transport has been shown to be inhibited by uncoupling agents with isolated mitochondria and a reconstituted malate-aspartate shuttle (29, 35, 36). Studies with rat heart mitochondria showed that aspartate efflux was energy dependent, being inhibited when energy production by oxidative phosphorylation was prevented (37, 38). As an alternative or additional mechanism, an energy dependence for the transport of various other anions across the mitochondrial membrane has been suggested (39-41). Thus, a high energy state of the mitochondrial membrane, or even a suitable proton or charge gradient (42, 43) may be required for anion transport via the malate-aspartate shuttle. Although one or more anion transport steps have been shown to be energy dependent, the energy requirements may be small and not proportional to flux. Furthermore, the β-hydroxybutyrate dehydrogenase substrate couple, as normally measured in total tissue extracts or in the mitochondrial incubation medium, may not provide a valid index of the intramitochondrial NAD oxidation-reduction state, even on the assumption of a single intramitochondrial NAD pool (44). On the basis of a lack of direct energy utilization for the malate-aspartate shuttle, and an ADP-controlled fully phosphorylating electron transport chain, NADH transport via the malate-aspartate shuttle should be associated with a lower oxygen consumption than NADH transport via the α-glycerophosphate shuttle due to the different P:O ratios for NAD- and flavin-linked substrates.

Recently, a third potentially important system for transport of NADH into mitochondria has been proposed (45) and investigated with a reconstituted mitochondrial system (46). This mechanism involves chain elongation of fatty acyl-CoA derivatives with the utilization of NADH in the extramitochondrial space, transfer of the fatty acyl-CoA into the mitochondrial matrix, followed by β oxidation to a shorter chain fatty acyl-CoA ester and its transport back into the extramitochondrial space for a further cycle of chain elongation. This system would be energetically favorable for inward transport of NADH because of the flavin step of β oxidation, but suffers from the disadvantage that β oxidation of long chain fatty acids, once initiated, is thought to proceed to completion (47, 48). As with the malate-aspartate shuttle, the fatty acid chain elongation cycle should be rotenone sensitive.

Data presented in this paper show that the rate of xylitol metabolism in rat liver is controlled by the rate of reoxidation of NADH in the cytosolic compartment and not by the capacity of xylitol dehydrogenase. This is most clearly seen from the large stimulation of glucose formation from xylitol induced by a variety of artificial electron acceptors. On the basis of previous work with a number of tissues this conclusion can probably be extended to other reduced substrates of cytosolic NAD-linked dehydrogenases such as ethanol or lactate (49-51). The large inhibition of calculated xylitol uptake observed after addition of 1 mM cyanide or antimycin A indicates that most of the NADH reoxidation in the absence of artificial electron acceptors is linked to mitochondrial respiration. The cyanide insensitive respiration accounted for the reoxidation of about 30 μmoles of NADH per 100 g rat weight per hour (Fig. 10), and could not be greatly stimulated by addition of artificial electron acceptors (Fig. 11). Control of the rate of NADH reoxidation in the cytosol by the rate of mitochondrial electron transport was also revealed by the stimulatory effect of low concentrations of the uncoupling agent 2,4-dinitrophenol, and addition of ornithine plus ammonia which induces an intramitochondrial utilization of ATP for citrulline synthesis. 2,4-Dinitrophenol has also been shown to increase ethanol utilization by rat liver slices (52) and rats in vivo (53), suggesting that the rate-limiting step of ethanol metabolism was the reoxidation of NADH to NAD rather than the capacity of the dehydrogenase. These authors also showed that most of the ethanol uptake by the rat liver slices was cyanide sensitive.

The incomplete inhibition of xylitol conversion to glucose by amobarbital suggests that part of the NADH transport into the mitochondria was mediated by the α-glycerophosphate shuttle. The data in Fig. 10 provide a value of 40 μmoles/100 g rat weight per hour for the flux, while Fig. 9 shows that xylitol addition in the presence of rotenone increased respiration by 57 pmols per oxygen per 100 g rat weight per hour. However, part of the respiratory increase may be accounted for by a stimulation of the cyanide insensitive respiration induced by the increased availability of cytosolic NADH after substrate addition. Earlier studies with rat liver mitochondria, slices or perfused rat liver (54-56) have indicated that the α-glycerophosphate shuttle has a very low capacity in this tissue, compared with the total rate of NADH transport, even when the mitochondrial α-glycerophosphate oxidase activity is increased many-fold, as in the hyperthyroid animal. A recent study of rates of [2-3H]glycerol from ADP to α-glycerophosphate oxidase, as is the case apparently with xylitol metabolism. However, even with xylitol as substrate, when tissue α-glycerophosphate concentrations rise to 12 mM (1), the strong competition observed between transport of NADH in mitochondria and extramitochondrial NAD dehydrogenases indicates the involvement of NAD-linked shuttles.

Operation of the fatty acid chain elongation shuttle, although unlikely, cannot be excluded from the present data. The initial inhibition of glucose production from xylitol by aminoxyacetate (Fig. 7), clearly shows participation of the malate-aspartate shuttle in the transport of reducing equivalents from the cytosol to the respiratory chain. Reversal of the inhibition after about 10 min is probably caused by an increased contribution from the α-glycerophosphate shuttle as the α-glycerophosphate levels rise. A similar substitution of increased flux through the α-glycerophosphate shuttle upon inhibition of the malate-aspartate shuttle, associated with increased tissue α-glycerophosphate levels, has been observed in rat hearts perfused with glucose (58). With lactate as substrate in perfused rat hearts, on the other hand, aminoxyacetate caused an 80% inhibition of lactate up-
take since there was a lack of precursors for α-glycerophosphate formation.

The greater inhibitory effect of β-hydroxybutyrate on glucose production from xylitol observed in Fig. 6, when flux was stimulated by ornithine and ammonia addition (cf. Figs. 1 and 2), suggests a greater contribution of the malate-aspartate shuttle possibly as a result of a change of the intramitochondrial pyridine nucleotides to a more oxidized state as availability of ADP increased due to the utilization of ATP for citrulline synthesis. The small increased rate of respiration observed after β-hydroxybutyrate addition, together with an increase in the state of reduction of the mitochondrial pyridine nucleotides, suggests the possibility that the electron transport chain may not be fully saturated with substrate during xylitol metabolism. The small effect of xylitol, and ethanol (56), on oxygen uptake by the liver indicates that NADH generation in the cytosol followed by transport to the mitochondria for oxidation can substitute fully saturated with substrate during xylitol metabolism. The energy requirements for operation of the hydrogen shuttles must be minimal.

Neither xylitol nor ethanol (1, 34) are capable of suppressing completely intramitochondrial generation of reducing equivalents by the citric acid cycle dehydrogenases indicating that transfer of reducing equivalents into the mitochondria as well as flux through the mitochondrial electron transport chain are both involved in the regulation of the utilization of the reduced substrate. The observation that β-hydroxybutyrate decreased gluconeogenesis from xylitol but increased it from lactate is of particular interest when the flux of anions across the mitochondrial membrane under the two conditions is taken into account. Transport of reducing equivalents via the malate-aspartate shuttle involves an influx of malate and glutamate and efflux of α-ketoglutarate and aspartate. Gluconeogenesis from lactate involves an influx of pyruvate and glutamate and efflux of α-ketoglutarate and aspartate. Since pyruvate carboxylase is the rate-limiting step for gluconeogenesis from lactate, the more reduced state of the mitochondrial NAD system after addition of β-hydroxybutyrate must cause an activation at this site, possibly by a decrease of the inhibitor acetoacetyl-CoA (59). The increased flux of anion transports probably follows from the increased rate of generation of mitochondrial oxalacetate. On the other hand, inhibition of glucose formation from xylitol by β-hydroxybutyrate probably involves an interaction between malate dehydrogenase and β-hydroxybutyrate dehydrogenase in the mitochondria and an inhibition principally of the malate aspartate shuttle, possibly by altering the malate gradient across the mitochondrial membrane (see also References 60, 61). Flux in the phosphorolytating electron transport chain, as controlled by the availability of phosphate acceptor, is a determinant of the intramitochondrial NAD oxidation-reduction state. It would appear, therefore, that transport of reducing equivalents into mitochondria via the malate-aspartate shuttle, being regulated by the mitochondrial NAD oxidation-reduction state, must also be regulated by the electron transport chain. Details of the factors affecting competition between the utilization of reducing equivalents delivered from the cytosol to the mitochondria and internally generated reducing equivalents from β oxidation and the reactions of the citric acid cycle require further evaluation, and are presumably determined by the control characteristics of the individual dehydrogenase and mitochondrial anion transport mechanisms.

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