Effects of Bicarbonate on Intercompartmental Reducing-Equivalent Translocation in Isolated Parenchymal Cells from Rat Liver

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1. Incubation of isolated liver cells in a medium containing bicarbonate raises malate concentrations almost sixfold compared with values obtained in a bicarbonate-free phosphate medium. The malate concentration of about 0.3 mM in bicarbonate medium is of the same order as the K_m for malate dehydrogenase. 2. The utilization of ethanol, glycerol and sorbitol was increased by 20–35% in bicarbonate medium. 3. Fluoromalate, a specific inhibitor of malate dehydrogenase and the malate carrier, inhibited or ethanol oxidation by 23%, glycerol uptake by 20% and sorbitol uptake by 42% in bicarbonate medium, but had a much smaller inhibitory action in phosphate medium. In consequence fluoromalate almost abolished the stimulatory effects of bicarbonate on substrate utilization. 4. Difluoro-oxaloacetate, a specific inhibitor of aspartate aminotransferase, had about one-half the inhibitory activity of fluoromalate. The two inhibitors in combination were less effective than fluoromalate by itself. 5. It is concluded that bicarbonate stimulates the utilization of reduced substrates, which are oxidized in the cytoplasmic compartment of the liver cell, by increasing the activity of rate-limiting malate dehydrogenase-dependent intercompartmental hydrogen shuttles. Both malate-oxaloacetate and malate-aspartate systems are involved in these hydrogen-translocation processes.

The likelihood has been recognized for a number of years that various shuttles are required to mediate the transfer of reducing equivalents from cytoplasm to mitochondria in the animal cell (Borst, 1963*a*; Lardy, 1966; Krebs, 1967; Greville, 1969). At least four distinct systems have been described, namely the malate-oxaloacetate (Lardy, 1966; Krebs, 1967), malate-aspartate (Borst, 1963*b*), glycerol 3-phosphate (Zebe *et al.*, 1959; Sacktor & Dick, 1962) and fatty acid (Whereat *et al.*, 1969) shuttles. Evidence has been adduced that some of these shuttles may operate in the hepatic metabolism of xylitol (Jakob *et al.*, 1971), ethanol (Williamson *et al.*, 1969), sorbitol (Berry *et al.*, 1973) and glycerol (Carnicero *et al.*, 1972; Berry *et al.*, 1973), but uncertainty remains as to the relative contributions of each shuttle for hepatic intercompartmental hydrogen translocation.

In studies on sorbitol and glycerol metabolism by intact isolated liver cells (Berry *et al.*, 1973), it was concluded that the glycerol 3-phosphate shuttle could account for up to 50% of the cytoplasmic reducing equivalents translocated during utilization of these substrates. Shuttles involving malate dehydrogenase appeared to mediate less than 15%

of the total reducing-equivalent translocation. However, these experiments were done by using a bicarbonate-free phosphate-buffered incubation medium in which the hepatic steady-state concentrations of dicarboxylic acids were likely to be decreased through diminished CO₂ fixation. In consequence, the contribution of malate dehydrogenase-dependent shuttles may have been artificially depressed. The role of malate dehydrogenase in hydrogen translocation was therefore re-examined by incubating isolated liver cells in a bicarbonate-saline medium and studying the inhibitory effects of the fluorodicarboxylic acids fluoromalate (Skilleter *et al.*, 1972) and difluoro-oxaloacetate (Kun *et al.*, 1963). The results of these experiments indicate that malate dehydrogenase-dependent shuttles may account for up to 45% of the reducing equivalents translocated during metabolism of ethanol, sorbitol or glycerol, and that both malate-oxaloacetate and malate-aspartate systems are operative in this hydrogen transfer.

Materials and Methods

Fluoro homologues of dicarboxylic acids were prepared by published methods (Kun & Dummel, 1969; Skilleter *et al.*, 1972). Collagenase and hyaluronidase were products of Worthington Biochemical Corp., Freehold, N.J., U.S.A. All other enzymes

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and cofactors were obtained from Boehringer Mannheim Corp., New York, N.Y., U.S.A. [$1\text{-}^{14}\text{C}$]-Ethanol (2mCi/mmol) was obtained from New England Nuclear Corp., Boston, Mass., U.S.A.

Male Sprague-Dawley rats weighing between 300 and 350g were used in these experiments. The animals were starved for 18h to deplete hepatic glycogen. Suspensions of isolated liver cells were prepared as described previously (Berry & Kun, 1972) by a modification of the method of Berry & Friend (1969). The cells were washed twice by centrifuging them at 50g for 2min and dispersing the pellet in about 20vol. of a medium containing 0.14M-NaCl, 5.4mM-KCl, 0.8mM-MgSO₄ and 0.8mM-sodium phosphate, adjusted to pH7.4. For studies using bicarbonate-free medium, cells were incubated in the same medium plus 2mM-CaCl₂ and 10mM-sodium phosphate buffer, pH7.4 ('phosphate medium'). The gas phase was 100% O₂ and the centre wells of the incubation vessels contained 0.2ml of 2M-KOH to absorb metabolic CO₂. For studies in the presence of bicarbonate the same incubation medium was used except that 25mM-NaHCO₃ replaced an equivalent quantity of NaCl ('bicarbonate medium') and KOH was omitted from the centre wells. In these experiments pH7.4 was maintained by using a gas phase of O₂+CO₂ (95:5). In most experiments the final volume of the incubation medium after cell addition was 4ml, but to conserve fluorodicarboxylic acids a final volume of 2ml was used in some instances. Incubations were carried out at 37°C for 40min with a wet weight of cells (Berry & Kun, 1972) of 90–150mg per vessel. Substrates were added at an initial concentration of 10mM.

After incubation 0.5ml of 4M-HClO₄ was added to each vessel and the precipitated protein removed by centrifuging. The extracts were neutralized with 2M-KOH and specific enzymic techniques were used to measure substrates and metabolites as described previously (Berry & Friend, 1969; Berry & Kun, 1972; Berry *et al.*, 1973). Malate was determined by a fluorimetric technique that uses the oxidation of malate to oxaloacetate (Williamson & Corkey, 1969). However, it was possible to dispense with hydrazine as the trapping agent for oxaloacetate, by addition to the cuvette of 200μmol of sodium glutamate and 5μl of aspartate aminotransferase (2mg of protein/ml). The reaction was thus driven to completion by converting the oxaloacetate formed into aspartate.

Recovery experiments demonstrated that a substantial amount of added ethanol was lost by evaporation. To overcome this difficulty in determining accurately the rate of ethanol utilization by the cells, the metabolism of [$1\text{-}^{14}\text{C}$]ethanol was examined. After incubation of the cells, 0.5ml of the neutralized perchlorate extract was transferred to a scintillation vial and 0.05ml of 2M-KOH was added. The contents of the vial were then evaporated to dryness at 70°C

and redissolved in 0.2ml of water. After addition of 10ml of scintillation mixture [4g of 2,5-diphenyloxazole and 200mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre of toluene] radioactivity was determined by liquid-scintillation counting. This procedure measured all metabolites of ethanol oxidized to the level of carboxylic acid, the major product being acetate (Lundquist *et al.*, 1962). Control studies showed that all unmetabolized ethanol was removed by this procedure. Moreover, it was found in several pilot experiments that less than 5% of the ethanol utilized was oxidized to CO₂ and incorporated into cell lipids as determined by extraction of the vessel contents by the Folch procedure (Folch *et al.*, 1957), or into glucose. Moreover, acetaldehyde was undetectable by enzymic assay (Bergmeyer, 1963). It was therefore concluded that determination of the labelled products of [$1\text{-}^{14}\text{C}$]ethanol metabolism provides a valid measurement of the rate of uptake of ethanol by liver cells from starved rats.

Assessment of rates of hydrogen flux from substrate to O₂

The flux of reducing equivalents from added substrate to O₂ was estimated by measuring the rate of utilization of ethanol, glycerol or sorbitol. The validity of this approach has been demonstrated previously (Berry *et al.*, 1973). For ethanol, 2mol of reducing equivalents are transferred for each mol of acetate formed. When sorbitol is the added substrate, a correction must be made for the accumulation of glycerol and glycerol 3-phosphate, whereas when glycerol is the added substrate, only the accumulation of glycerol 3-phosphate need be taken into account. After appropriate correction, the rates of sorbitol or glycerol uptake are equivalent to the rates of hydrogen flux from these substrates to O₂ (Berry *et al.*, 1973).

Hydrogen flux from sorbitol or glycerol to O₂ can also be assessed by measuring the accumulation of the major products of substrate metabolism, namely glucose and lactate. This is because these products cannot be formed from sorbitol or glycerol without transfer of reducing equivalents from substrate to O₂. This approach gives similar results to those obtained with the present method (Berry *et al.*, 1973) and may be used to check the data in Tables 1 and 3.

Results

Concentrations of malate were measured in perchlorate extracts of cells and medium from starved rats after incubation of the cells with ethanol. For five experiments in phosphate medium the concentration of malate found was 54 ± 25 nmol/g of liver, and 284 ± 34 nmol/g of liver for eight experiments in bicarbonate medium. Since this malate was formed

Table 1. Rates of utilization of glycerol and sorbitol by isolated liver cells

Cells from starved rats were incubated in a phosphate-buffered medium containing 0.14M-NaCl, 5.4mM-KCl, 0.8mM-MgSO₄, 2mM-CaCl₂ and 10mM-sodium phosphate, pH 7.4, with a gas phase of 100% O₂, or in a bicarbonate-buffered medium containing 0.115M-NaCl, 5.4mM-KCl, 0.8mM-MgSO₄, 2mM-CaCl₂, 10mM-sodium phosphate and 25mM-NaHCO₃. Bicarbonate medium was maintained at pH 7.4 by gassing with O₂+CO₂ (95:5). Substrates were added at an initial concentration of 10mM. The final volume was 4ml and incubations were carried out for 40min at 37°C. Results, expressed as $\mu\text{mol}/\text{min}$ per g wet wt. of liver, are means $\pm$ S.E.M. of five experiments.

Added substrate	Medium	Metabolic changes					Rate of re- ducing-equa- lent flux to O ₂
		Glucose	Fructose	Lactate	Glycerol 3-phosphate	Glycerol	
Glycerol	Phosphate	0.52 $\pm$ 0.04	—	0.10 $\pm$ 0.02	0.32 $\pm$ 0.02	-1.40 $\pm$ 0.04	1.08 $\pm$ 0.03
Glycerol	Bicarbonate	0.70 $\pm$ 0.02	—	0.15 $\pm$ 0.02	0.28 $\pm$ 0.02	-1.70 $\pm$ 0.03	1.42 $\pm$ 0.05
Sorbitol	Phosphate	1.38 $\pm$ 0.07	0.13 $\pm$ 0.05	0.23 $\pm$ 0.03	0.12 $\pm$ 0.02	0.04 $\pm$ 0.01	1.50 $\pm$ 0.07
Sorbitol	Bicarbonate	1.79 $\pm$ 0.10	0.16 $\pm$ 0.03	0.21 $\pm$ 0.03	0.13 $\pm$ 0.01	0.04 $\pm$ 0.01	1.85 $\pm$ 0.09
						-1.66 $\pm$ 0.07	
						-2.02 $\pm$ 0.08	

by metabolic processes it is likely that it remained within the cells (Hems *et al.*, 1968). Thus the intra-cellular steady-state concentration of malate was almost six times as great for cells incubated in bicarbonate medium, presumably because more CO₂ was available for dicarboxylic acid formation under these conditions. Similar measurements were carried out with sorbitol or glycerol as added substrate. For two experiments with each substrate in bicarbonate medium the average malate concentration was 110nmol/g of liver for sorbitol and 70nmol/g of liver for glycerol. However, malate concentrations in perchlorate extracts derived from cells in phosphate medium were immeasurably low.

The rates of utilization of ethanol, glycerol and sorbitol by liver cells from starved rats were compared in the absence and presence of bicarbonate. In six paired experiments ethanol oxidation by the cells was 0.64 $\pm$ 0.08 $\mu\text{mol}/\text{min}$ per g of liver in phosphate medium and 0.81 $\pm$ 0.09 $\mu\text{mol}/\text{min}$ per g of liver in bicarbonate medium ($P < 0.001$ by Student's *t* test for paired results). Metabolic-balance studies of glycerol and sorbitol utilization are recorded in Table 1. The rates of glucose formation, substrate uptake and flux of reducing equivalents from substrate to O₂ were all significantly greater in the presence of bicarbonate. Thus in bicarbonate medium glucose formation from glycerol was increased by 35%, glycerol uptake by 20% and flux of reducing equivalents from glycerol to O₂ by 31%. For all these values $P < 0.01$ by Student's *t* test for unpaired results. Likewise, glucose formation from sorbitol was increased by 30%, sorbitol uptake by 22% and flux of reducing equivalents from sorbitol to O₂ by 23% in bicarbonate medium. Again $P < 0.01$ for these values by Student's *t* test for unpaired results.

This correlation between higher intracellular malate concentration and a greater rate of substrate utilization in bicarbonate medium suggested the possibility that hydrogen shuttles involving malate dehydrogenase might play a more important role in the transfer of reducing equivalents from ethanol, glycerol or sorbitol to O₂ when the availability of CO₂ was not limiting. Direct evidence for this possibility was sought by the use of fluorodicarboxylic acids. Fluoromalate, a potent inhibitor of malate dehydrogenase and the malate carrier (Skilleter *et al.*, 1972), was used to assess the contribution of shuttles involving this enzyme, and difluoro-oxaloacetate, an inhibitor of aspartate aminotransferase (Kun *et al.*, 1963), to evaluate the role of the malate-aspartate system. The dose-response curves in Fig. 1 showed little increase in inhibition of utilization of ethanol, glycerol or sorbitol over an inhibitor concentration range from 2.5 to 10mM. To conserve fluorodicarboxylic acids, 5mM concentrations were used as a routine in subsequent experiments.

Fig. 1. Effects of fluorodicarboxylic acid concentration on rates of substrate utilization by isolated liver cells

Isolated liver cells were incubated in bicarbonate medium in the presence of fluoromalate (—) or difluoro-oxaloacetate (---) at the concentrations indicated. The added substrates (10mM initial concentration) were ethanol (Δ), glycerol ($\bullet$) or sorbitol ($\circ$). The incubation time was 40min and the cell mass was 140mg wet wt. per vessel.

The effects of fluorodicarboxylic acids on ethanol metabolism are recorded in Table 2. In phosphate medium the effects were small, the inhibition of ethanol uptake by fluoromalate or difluoro-oxaloacetate being less than 10%. In bicarbonate medium, however, the effects of the inhibitors were more marked and the mean inhibition by fluoromalate was 23% ($P < 0.01$ by Student's t test for paired results). The inhibition by difluoro-oxaloacetate averaged 12% and the combination of inhibitors was slightly less effective than fluoromalate by itself. Analogous results were obtained in studies of the effects of fluorodicarboxylic acids on utilization of glycerol and sorbitol (Table 3). As found previously (Berry *et al.*, 1973) the effects of these inhibitors in phosphate medium were relatively small. However, in bicarbonate medium fluoromalate inhibited glucose formation from glycerol by 29%, glycerol uptake by 20% and flux of reducing equivalents from glycerol to O_2 by 26% ($P < 0.01$ by Student's t test for unpaired results). Difluoro-oxaloacetate was considerably less inhibitory (Table 3) and the inhibitors in combination were again somewhat less effective than fluoromalate added by itself. This latter phenomenon is most probably explained by the fact that both difluoro-oxaloacetate and fluoromalate are recognized as substrate homologues of malate dehydrogenase (Kun *et al.*, 1963) and if present simultaneously may compete for the enzymic site. Difluoro-oxaloacetate does not inhibit malate dehydrogenase and therefore counteracts the inhibitory effect of fluoromalate.

The effects of fluorodicarboxylic acids were even more pronounced when sorbitol was the added substrate. Fluoromalate inhibited glucose formation by cells incubated in bicarbonate medium by an average of 43% (Table 3), substrate uptake by 42%

Table 2. Effects of fluorodicarboxylic acids on ethanol utilization by isolated liver cells

Liver cells from starved rats were incubated in phosphate medium or bicarbonate medium in the presence of fluoromalate and difluoro-oxaloacetate at a concentration of 5mM and ethanol (10mM) as the added substrate. Other experimental conditions were as for Table 1.

Medium	Inhibitor	No. of expts.	Rate of ethanol utilization ($\mu\text{mol/min per g of liver}$)	Inhibition (%)
Phosphate	None	2	0.72	—
Phosphate	Fluoromalate	2	0.68	6
Phosphate	Difluoro-oxaloacetate	2	0.66	9
Phosphate	Fluoromalate, difluoro-oxaloacetate	2	0.66	9
Bicarbonate	None	6	0.81 ± 0.04	—
Bicarbonate	Fluoromalate	6	0.59 ± 0.03	23
Bicarbonate	Difluoro-oxaloacetate	4	0.71 ± 0.04	12
Bicarbonate	Fluoromalate, difluoro-oxaloacetate	4	0.64 ± 0.04	21

Table 3. *Effects of fluorodicarboxylic acids on utilization of glycerol and sorbitol by isolated liver cells*

Liver cells from starved rats were incubated in phosphate medium or bicarbonate medium in the presence of fluoromalate and difluoro-oxaloacetate at a concentration of 5mm with glycerol or sorbitol added at an initial concentration of 10mm. Other experimental conditions were as for Table 1. Rates are expressed as $\mu\text{mol/min}$ per g of liver.

Medium	Substrate	Inhibitor	No. of expts.	Metabolic changes					Reducing-equivalent flux to O_2
				Glucose	Fructose	Lactate	Glycerol 3-phosphate	Glycerol	Sorbitol
Phosphate	Glycerol	None	2	0.58	—	0.12	0.35	-1.44	—
Phosphate	Glycerol	Fluoromalate	2	0.53	—	0.24	0.32	-1.49	—
Phosphate	Glycerol	Difluoro-oxaloacetate	2	0.55	—	0.15	0.30	-1.40	—
Phosphate	Glycerol	Fluoromalate, difluoro-oxaloacetate	2	0.54	—	0.22	0.31	-1.35	—
Bicarbonate	Glycerol	None	4	0.70 ± 0.03	—	0.15 ± 0.02	0.30 ± 0.02	-1.69 ± 0.04	—
Bicarbonate	Glycerol	Fluoromalate	4	0.50 ± 0.02	—	0.27 ± 0.05	0.31 ± 0.01	-1.36 ± 0.03	—
Bicarbonate	Glycerol	Difluoro-oxaloacetate	4	0.63 ± 0.01	—	0.19 ± 0.03	0.29 ± 0.01	-1.53 ± 0.06	—
Bicarbonate	Glycerol	Fluoromalate, difluoro-oxaloacetate	4	0.54 ± 0.02	—	0.25 ± 0.02	0.26 ± 0.02	-1.40 ± 0.04	—
Phosphate	Sorbitol	None	2	1.55	0.09	0.26	0.12	0.03	-1.80
Phosphate	Sorbitol	Fluoromalate	2	1.22	0.14	0.43	0.13	0.04	-1.38
Phosphate	Sorbitol	Difluoro-oxaloacetate	2	1.42	0.09	0.32	0.14	0.04	-1.68
Phosphate	Sorbitol	Fluoromalate, difluoro-oxaloacetate	2	1.28	0.10	0.36	0.17	-0.04	-1.43
Bicarbonate	Sorbitol	None	3	1.93	0.13	0.23	0.12	0.03	-2.12
Bicarbonate	Sorbitol	Fluoromalate	3	1.10	0.14	0.44	0.17	0.03	-1.24
Bicarbonate	Sorbitol	Difluoro-oxaloacetate	3	1.61	0.12	0.29	0.12	0.04	-1.75
Bicarbonate	Sorbitol	Fluoromalate, difluoro-oxaloacetate	3	1.19	0.12	0.37	0.15	0.04	-1.45

and hydrogen flux from sorbitol to O_2 by 47%. The inhibitory action of difluoro-oxaloacetate was about one-half that of fluoromalate and the effects of the two inhibitors in combination were again less than fluoromalate added by itself.

Discussion

In these experiments the concentrations of malate were of the same order as the K_m for malate dehydrogenase, which is about 0.2 mM (Kun *et al.*, 1963), when liver cells were incubated in bicarbonate medium and were well below this value when the cells were suspended in a bicarbonate-free phosphate medium. It was also observed that utilization of ethanol, glycerol and sorbitol was significantly higher when cells were suspended in bicarbonate medium than when incubated in the absence of bicarbonate. This suggested a possible correlation between the activity of malate dehydrogenase and the rate of cellular substrate uptake. However, a direct role for malate dehydrogenase has not been established on any of the metabolic pathways studied here, namely the oxidation of ethanol to acetate and the conversion of glycerol or sorbitol into glucose and lactate. On the other hand, involvement of malate dehydrogenase in intercompartmental hydrogen translocation is now well accepted (Borst, 1963a; Lardy, 1966; Krebs, 1967; Greville, 1969; Berry & Kun, 1972). We therefore conclude that the stimulation of the uptake of reduced substrates in bicarbonate medium was related to an increased activity of rate-limiting intercompartmental hydrogen shuttles involving malate dehydrogenase. We attribute the enhanced activity of these shuttles to an increased steady-state concentration of malate, which in turn was a consequence of a stimulated CO_2 fixation by liver cells incubated in bicarbonate medium. The relatively high K_m (1 mM) for bicarbonate of pyruvate carboxylase (Keech & Utter, 1963) adds credence to these views.

Support for these conclusions came from studies with fluoromalate. It was shown previously (Berry & Kun, 1972) that fluoromalate in a cellular system does not inhibit mitochondrial oxidations because its penetration into mitochondria is slow (Skilleter *et al.*, 1972). Its effects are therefore not related to a non-specific inhibition of mitochondrial metabolism but rather to an action on processes involving cytoplasmic malate metabolism and transport. In the present study fluoromalate inhibited sorbitol utilization by cells incubated in bicarbonate medium by over 40% and caused a somewhat similar inhibition of uptake of glycerol or ethanol. The effects of this inhibitor in phosphate medium were considerably less, so that rates of substrate utilization in phosphate medium were rather similar to the rates in bicarbonate medium in the presence of fluoromalate. In other words,

fluoromalate abolished the stimulatory effects of bicarbonate on substrate uptake, thereby providing corroborative evidence that these stimulatory effects were mediated through processes involving malate dehydrogenase.

It is noteworthy that fluoromalate has no effect on fructose utilization (Berry & Kun, 1972), nor does bicarbonate medium stimulate conversion of fructose into glucose and lactate (M. N. Berry, unpublished work). Gluconeogenesis and lactate formation from fructose do not require intercompartmental hydrogen translocation, whereas the transfer of reducing equivalents from substrate to O_2 is an essential feature of metabolism of ethanol, glycerol and sorbitol. Thus there is a clear correlation between the requirement for hydrogen translocation in the metabolism of a substrate and the stimulation of its uptake in bicarbonate medium or the inhibition of its utilization by fluoromalate.

A distinction between malate-oxaloacetate and malate-aspartate shuttles could be made by the differential effects of fluoromalate and difluoro-oxaloacetate, the latter inhibiting transamination only. Since the maximal inhibition of substrate uptake by difluoro-oxaloacetate was about half that of fluoromalate it is likely that at least half of the malate dehydrogenase-dependent hydrogen translocation was mediated by a malate-oxaloacetate shuttle and the remainder by the malate-aspartate system. Presumably specific carriers exist within the mitochondrial membrane to transfer aspartate or oxaloacetate, as the case may be (Greville, 1969).

An assessment of the contribution of the various shuttles in the transfer of hydrogen from sorbitol and glycerol to O_2 can be made on the basis of the degree of inhibition of substrate uptake by antimycin, rotenone and the fluorodicarboxylic acids (Berry *et al.*, 1973). Our revised estimates based on these current studies using bicarbonate medium do not differ greatly from previous calculations, except that the significance of malate dehydrogenase-dependent shuttles is upgraded. The rate of hydrogen transfer from sorbitol to O_2 is about $1.8 \mu\text{mol/min per g}$ of liver, and it is inferred that about 45% of the hydrogen is translocated by shuttles involving malate dehydrogenase. An equal amount passes to O_2 by systems involving a rotenone-insensitive antimycin-sensitive system, presumably the glycerol 3-phosphate shuttle (Berry *et al.*, 1973). In studies with thyroxine-treated rats (Werner & Berry, 1974) it was found that shuttles mediated by malate dehydrogenase make a much smaller contribution, evidently owing to the stimulation by thyroxine treatment of the biosynthesis of hepatic mitochondrial glycerol 3-phosphate dehydrogenase in the rat (Lee & Lardy, 1965).

The uptake of ethanol by liver cells in bicarbonate medium represents a rate of transfer of reducing equivalents to O_2 of about $1.6 \mu\text{mol/min per g}$ of liver.

However, the degree of intercompartmental hydrogen translocation involved in this ethanol oxidation is difficult to assess at present because of uncertainty over the site of oxidation of acetaldehyde (Marjanen, 1972) and the possible existence of a system within the endoplasmic reticulum for the oxidation of ethanol (Lieber & DeCarli, 1968).

It should be pointed out that the rates of sorbitol utilization observed in cells incubated in bicarbonate medium in the present series of experiments were not much higher than in our previous studies where only phosphate medium was used (Berry *et al.*, 1973). This is probably explained by the greater concentrations of cells used in the earlier work, leading to higher steady-state concentrations of metabolic CO₂ within the incubation medium. On the other hand, the rates of cellular glycerol uptake reported in the present work are substantially lower than earlier data indicate (Berry *et al.*, 1973). This is because in the present study the rate of glycerol uptake is based on an incubation period of 40min, whereas the earlier measurements were derived from incubations of only 10min duration. The marked decline in rates of glycerol uptake during prolonged incubation of the cells (Berry *et al.*, 1973), presumably owing to product inhibition (Robinson & Newsholme, 1969), accounts for these discrepancies.

The observation that CO₂ stimulates hepatic glycerol utilization is not new, but confirms previous studies by Longmore *et al.* (1969) performed with isolated perfused rat liver. The present results show that the uptake of other reduced substrates such as ethanol and sorbitol are also increased by CO₂. The stimulation in the activity of malate dehydrogenase-dependent hydrogen shuttles by bicarbonate provides a plausible explanation for these phenomena. The sensitivity of substrate utilization to agents which alter the activity of enzymic components of the hydrogen-shuttle systems provides corroborative evidence for the rate-limiting functions of hydrogen shuttles in the metabolism of reduced substrates by intact hepatic cells.

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