Interference of Ethanol Oxidation with Gluconeogenesis in the Perfused Guinea Pig Liver*

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ABSTRACT: In perfused guinea pig livers, pyruvate is less rapidly converted into glucose than is lactate. It is shown that the conversion of 3-phosphoglycerate to 3-phosphoglyceraldehyde is the rate-limiting step. Ethanol oxidation supplied the cytosol with free NADH and reversed the pyruvate inhibition. With lactate as the gluconeogenic precursor ethanol oxidation produced a surplus of reducing equivalents which prevents malate and glycerol 1-phosphate from being oxidized to glucose precursors resulting in a 70% inhibition of gluconeogenesis. In the presence of alanine, glucose formation is suppressed only 30% by ethanol. Therefore, in the perfused guinea pig liver the rate of gluconeogenesis seems to be dependent only on the relation between availability of hydrogen acceptors and production of reducing equivalents according to the hypothesis of Freinkel *et al.* (Freinkel, N., Cohen, A. F., Arky, R. A., and Foster, A. E. (1965), *J. Clin. Endocrinol. Metab. 25*, 76).

In the presence of ethanol phenethylbiguanide led to a marked accumulation of reducing equivalents in the perfused guinea pig liver causing a 300% rise of the lactate, malate, and glycerol 1-phosphate concentrations. Even under these conditions, when cell respiration was inhibited, no further control at the pyruvate carboxylase or phosphofructokinase reactions could be detected from the pattern of hepatic metabolite concentrations.

During ethanol oxidation the hepatic redox pair of the phosphopyridine nucleotides becomes more reduced than is usual (Smith and Newman, 1959; Forsander *et al.*, 1965; Freinkel *et al.*, 1965). It is now generally believed that the increase in NADH drives the dehydrogenases to trap gluconeogenic precursors from the well-known pathway, which converts pyruvate to glucose. Freinkel *et al.* (1965) proposed an additional mechanism which in the rat liver may be the primary one. Increase of intramitochondrial NADH would decrease fatty acid oxidation, resulting in an inhibition of the pyruvate carboxylase by a lack of acetyl coenzyme A. This concept was supported by Kreisberg (1967) who showed that, in the isolated perfused rat liver, inhibition of gluconeogenesis is more related to a decrease in ketone body formation than to an increase of NADH. This correlation could not be found in the perfused guinea pig liver, because 10 mM ethanol lowered glucose formation from alanine, whereas the ketone bodies were elevated. Glucose formation in the presence of lactate was more suppressed by 10 mM ethanol than in the presence of alanine. However, gluconeogenesis from pyruvate was stimulated by ethanol; therefore, in the perfused guinea pig liver, the influence of ethanol seemed to be dependent only upon the concentration of the hydrogen acceptors and the amount of free NADH produced in the cytosol.[1] Sandler *et al.* (1967) reported a synergistic effect of DBI and ethanol on gluconeogenesis. These authors observed that 0.8 mM DBI[2] enhanced the suppression of the formation of labeled CO_2 and glucose from [^{14}C]alanine by 10 mM ethanol in rabbit liver slices. Jangaard *et al.* (1968) proposed that the inhibition of cell respiration by DBI affects pyruvate oxidation. This could lower the acetyl-CoA concentration required for pyruvate carboxylation. The increase of ADP by DBI could have an additional inhibitory effect on the conversion of pyruvate into oxalacetate according to Walter *et al.* (1966). Therefore, the combined administration of ethanol and DBI should exert a synergistic effect on the pyruvate carboxylation leading to a significant decrease of the malate concentration. However, in the presence of DBI ethanol led to a threefold elevation of the lactate, malate, and glycerol 1-phosphate concentrations manifesting a marked accumulation of reducing equivalents. Even under these conditions, when cell respiration was inhibited as indicated by a drop in the ATP/ADP ratio, the concentrations of the ketone bodies were not altered.

Materials and Methods

Livers were obtained from male, albino guinea pigs (Random Breed, Scientific Small Animal Farm and Lab-

* From the Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin 53706. *Received June 4, 1968.* Supported by grants from the Life Insurance Medical Research Fund and National Institutes of Health.

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[1] Cytosol refers specifically to the cytoplasm minus mitochondria and endoplasmic reticulum components (Lardy, 1965).

[2] Abbreviations used in this work that are not listed in *Biochemistry 5*, 1445 (1966), are: PEP, phosphoenolpyruvate; 2-PGA, 2-phosphoglycerate; 3-PGA, 3-phosphoglycerate; GAP, 3-phosphoglyceraldehyde; DAP, dihydroxyacetone phosphate; DBI, phenethylbiguanide; CoA, coenzyme A.

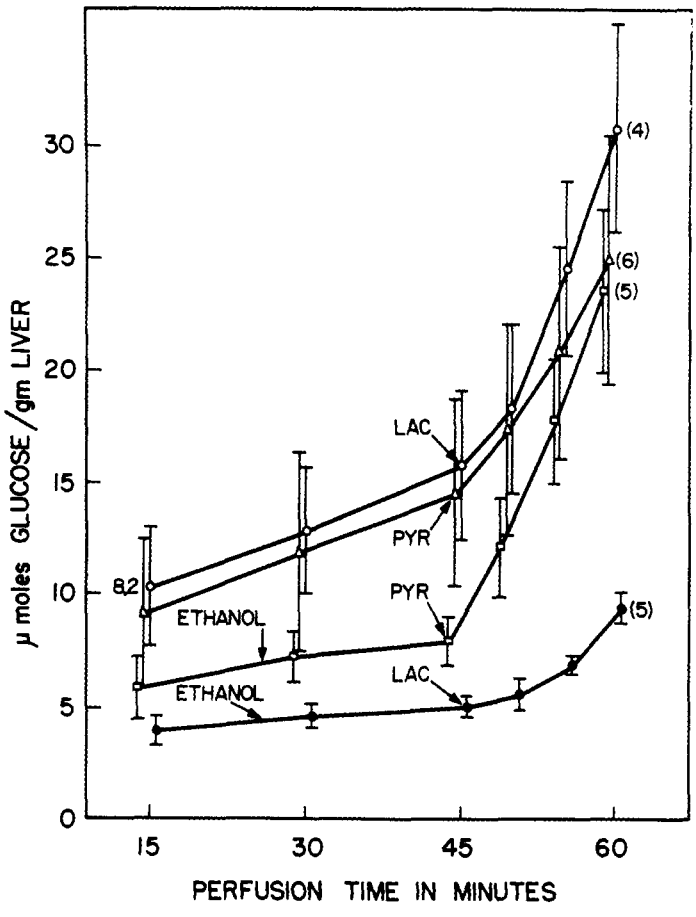

FIGURE 1: Glucose appearance in the perfusion medium in the presence and absence of ethanol. The addition of lactate (10 mM), pyruvate (10 mM), and ethanol (10 mM) are marked by arrows. The number of perfusions contributing to each line is given in parentheses. The vertical bars represent standard deviations. The true value is 8.2 for the first point, if a line is shifted parallel to its original position.

oratories Inc., Chicago, Ill.) weighing 280–350 g after having been fasted for about 24 hr. The perfusion technique used was that of Brauer *et al.* (1951), as previously described by Veneziale *et al.* (1967). The perfusion medium contained 3 g % bovine albumin (fraction V, Armour Pharmaceutical Corp.), 0.5 mg of sodium ampicillin (Polycillin-N, Bristol Laboratories, Syracuse, N. Y.), and erythrocytes from 60 ml of guinea pig blood obtained by heart puncture, washed twice, and taken up in Krebs–Ringer bicarbonate buffer to 120 ml. Heparin was omitted from the medium. After the liver was isolated and connected with the perfusion medium, the first 20 ml of the venous outflow was discarded, so that the final volume of the circulating medium was 100 ml during the perfusion. The flow rate of the perfusion medium was 18–24 ml/min. Ethanol (Rosville Gold Shield Alcohol, Commercial Solvents Corp., Terre Haute, Ind.) was added after 25 min, the substrates after 45 min. After 60-min perfusion liver samples were taken by the tissue freezing method (Wollenberger *et al.*, 1958). During this time 4.7 ± 0.4 μl/min per g (17)[3] of bile were produced.

All hepatic metabolites were determined enzymatically as described in Bergmeyer (1963) the same day the extracts were prepared. Acetoacetate and β-hydroxybutyrate were measured according to Williamson *et al.* (1962), citrate by the method of Gruber and Moellering (1966), ATP according to Lamprecht and Trautscholt (Bergmeyer, 1963) and blood glucose by the glucostat method (Worthington Biochemical Corp.). When the rates of gluconeogenesis from different precursors were compared with each other, the glucose produced over the last 15-min period was calculated and the endogenous rate during the 15 min before the addition of the substrate was subtracted.

Liver mitochondria from guinea pigs fasted 24 hr were isolated by a modification (Johnson and Lardy, 1967) of the method of Schneider (1948). The incubation medium contained 15 mM KCl, 2 mM ATP, 2 mM $MgCl_2$, 12 mM PO_4^{2-}, about 200 mM sucrose, 10 mM pyruvic acid, and 0.5 ml of mitochondrial suspension in a final volume of 3 ml. The pH of the phosphate and the pyruvic acid solutions was adjusted to 7.4 with triethanolamine. After 8 min of thermal equilibration at 30°, the reaction was started by the addition of 0.2 ml of glucose (0.25 M) and hexokinase (grade III, Sigma Chemical Corp., St. Louis, Mo.; 2.5 mg/1.0 ml). Acetaldehyde (Mallinckrodt Chemical Works, St. Louis, Mo.) was distilled twice prior to use. The statistical evaluation of the changes in the ATP/ADP ratio after ethanol administration in comparison with the control values was performed by means of the analysis of variance (F test).

Results

Gluconeogenesis from Various Precursors during Ethanol Oxidation. Glucose formation from pyruvate as

[3] Number in parentheses indicates the number of observations.

TABLE I: Hepatic Concentrations of the Ketone Bodies after 60-min of Perfusion.

Substrate[a] Ethanol	Pyr −	Pyr +	Lac −	Lac +	Ala −	Ala +
β-OH-butyrate	430 ± 90 (7)[b]	460 ± 140 (5)	440 ± 110 (6)	680 ± 110 (6)	410 ± 80 (8)	690 ± 170 (5)
Acetoacetate	1070 ± 260 (7)	980 ± 310 (5)	1110 ± 290 (6)	1070 ± 300 (8)	1060 ± 320 (8)	1350 ± 80 (5)
Ketone bodies	1490 ± 330 (7)	1430 ± 440 (5)	1530 ± 370 (6)	1750 ± 340 (6)	1470 ± 310 (8)	2040 ± 120 (5)
β-OH-But/Ac-ac[c]	0.40 ± 0.06 (7)	0.47 ± 0.05 (5)	0.39 ± 0.08 (6)	0.63 ± 0.22 (6)	0.39 ± 0.11 (8)	0.51 ± 0.15 (5)
Lac/Pyr	0.5 ± 0.1 (7)	1.0 ± 0.3 (5)	21.5 ± 6.7 (6)	44.6 ± 6.7 (6)	5.9 ± 2.2 (6)	8.0 ± 2.6 (5)

[a] The substrates and ethanol were added as indicated in Figure 1 and 2. [b] The concentrations are given in nanomoles per gram wet weight with standard deviations and the numbers of the contributing values. [c] β-Hydroxybutyrate/acetoacetate.

substrate was about 30% less than from lactate (Figure 1) in the absence of ethanol. After the addition of ethanol, pyruvate was converted into glucose as fast as lactate; the total amount of glucose formed from pyruvate was even higher than from lactate if the endogenous gluconeogenesis, which was completely depressed by ethanol, is subtracted. Under the same experimental conditions ethanol suppressed gluconeogenesis 70% with lactate as substrate (Figure 1), and 30% with alanine (Figure 2).

Concentrations of Hepatic Metabolites during Ethanol Oxidation. In Figure 3a,b the concentrations of the gluconeogenic metabolites from lactate to glucose 6-phosphate are compared under various conditions. The control values of 100% represent the concentrations of hepatic metabolites when lactate was added to the perfusion medium in the absence of ethanol. With pyruvate (Figure 3a, solid circles) 3-phosphoglycerate was increased more than 500% and all metabolites beyond 3-phosphoglycerate were lowered. This crossover phenomenon was reversed to some extent by ethanol (Figure 3a, solid triangles). Glucose 6-phosphate reached the control value in agreement with the fact that in the presence of ethanol pyruvate was converted into glucose at the same rate as lactate.

When lactate and ethanol were combined, the level of 3-phosphoglycerate drops to less than 10% of the control value. Malate and α-glycerophosphate both are elevated more than 200% (Figure 3b). A similar pattern of hepatic metabolite concentration was obtained with alanine as substrate in the presence of ethanol (Figure 4).

In Table I the lactate/pyruvate and the β-hydroxybutyrate/acetoacetate ratios are compared for different conditions. In the absence of ethanol the β-hydroxybutyrate/acetoacetate ratio was constant at 0.40 and independent of the extramitochondrial NADH level. Although the lactate/pyruvate ratio cannot be used for the estimation of free NADH if lactate or pyruvate is added to the medium, the level of free NADH can be expected to vary according to the change in the 3-phosphoglycerate concentration. This shows that compartmental differences in the redox potentials are not compensated during a short time period. Only under extreme conditions, when lactate and ethanol together caused a great surplus in reducing equivalents the β-hydroxybutyrate/acetoacetate ratio was driven to a slightly more reduced state.

The ketone body formation was not affected by ethanol with pyruvate and lactate (Table I), but significantly increased with alanine. This elevation of the ketone body concentration was recorded 15 min after the addition of alanine and could no longer be detected after a further perfusion period of 30 min (Table II).

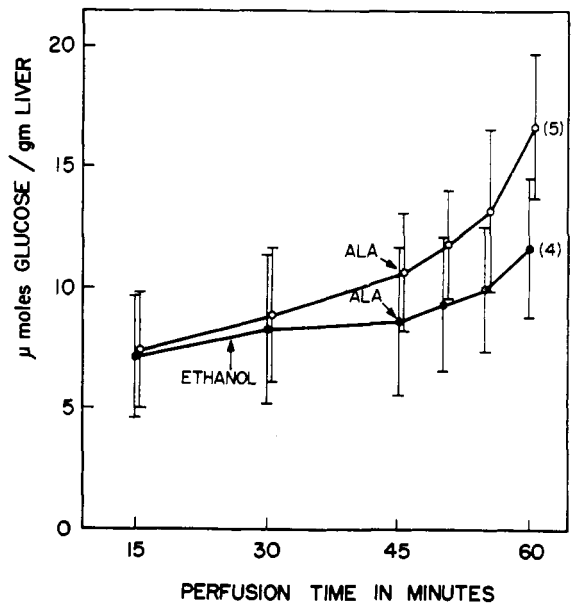

FIGURE 2: Glucose formation from alanine (10 mM) with and without ethanol (10 mM). See the legend to Figure 1 for explanation of symbols.

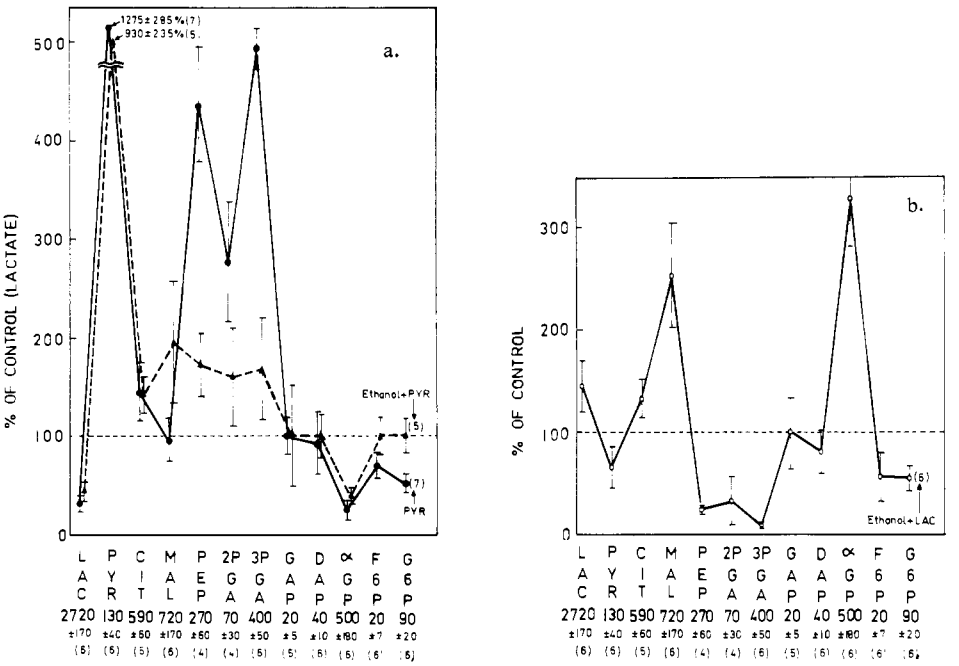

FIGURE 3: Ethanol influence on gluconeogenic metabolites. (a) The pattern of gluconeogenic metabolites with lactate and pyruvate as substrate and its alteration by ethanol. Control values given in nmoles per gram wet weight at the bottom of the figure together with standard deviations and number of experiments (in parentheses) were obtained when 10 mM lactate was used as substrate. The curves with solid circles (in the absence of ethanol) and with solid triangles (with 10 mM ethanol) were obtained by the addition of 10 mM pyruvate. The vertical lines indicate standard deviations of the means. The number of perfusions contributing to the experimental data are given in parentheses on the right end of the curve and are valid for each point if not stated otherwise. Ethanol and substrates were added to the perfusion medium as indicated in Figure 2. (b) Influence of ethanol on metabolite formation in the presence of lactate. The control values were obtained in the absence of ethanol.

TABLE II: Hepatic Concentrations of the Ketone Bodies after 90-min Perfusion (Substrate, Alanine).

Ethanol (10 mM)	−	−	−	+
DBI (0.08 mM)	−	−	+	+
β-Hydroxybutyrate	480[a] ± 100 (5)	790 ± 90 (4)	730 ± 60 (5)	1280 ± 110 (5)
Acetoacetate	1230 ± 240 (5)	1040 ± 110 (4)	980 ± 180 (5)	440 ± 140 (5)
Ketone bodies	1710 ± 320 (5)	1830 ± 180 (4)	1710 ± 210 (5)	1710 ± 220 (5)
β-Hydroxybutyrate/acetoacetate	0.39 ± 0.07 (5)	0.76 ± 0.07 (4)	0.75 ± 0.11 (5)	2.91 ± 0.98 (5)

[a] The concentrations are given in nanomoles per gram wet weight with standard deviations and the numbers of the contributing values.

The pattern of glycolytic metabolite concentrations was not altered during these additional 30 min as can be seen by comparing Figure 4 with Figure 6a.

The ATP/ADP ratio was slightly ($P < 0.05$) increased 60 min after the addition of ethanol in the presence of alanine (Table III). This could be due to oxidation of acetate derived from ethanol. In Table IV it is shown that acetate enhanced the oxygen uptake by isolated liver mitochondria in the presence of pyruvate. With acetate alone no significant oxygen consumption was obtained. The mitochondrial respiration was not affected by ethanol, but inhibited by acetaldehyde, which is also formed during ethanol oxidation, but usually rapidly converted into acetate in the intact liver. High concentrations of acetaldehyde blocked the oxygen uptake by the mitochondria completely, if pyruvate, malate, β-hydroxybutyrate, or glutamate were used as substrate, but not with succinate. Kiessling (1963) first re-

TABLE III: Influence of Ethanol and of DBI on the Hepatic ATP/ADP Ratio.[a]

Ethanol (10 mM)	−	+	−	+
DBI (0.08 mM)	−	−	+	+
ATP	2080 ± 230 (5)	2010[b] ± 140 (6)	1870 ± 280 (4)	1770 ± 170 (6)
ADP	530 ± 130 (5)	440 ± 50 (6)	810 ± 90 (4)	700 ± 80 (6)
ATP/ADP	3.9 ± 0.7 (6)	4.6 ± 0.2 (6)		2.5 ± 0.3 (6)

[a] Determined in liver sample taken after 90-min perfusion. Ethanol and DBI were added to the perfusion medium as indicated in Figure 5. [b] The concentrations are given in nanomoles per gram wet weight with standard deviations and numbers of the contributing values.

ported a marked effect of acetaldehyde on respiration in rat liver mitochondria using pyruvate as substrate in the presence of malate. Acetaldehyde (2.0 mM) led to slight consumption of oxygen in the absence of other substrates, as was also observed by Walhenstein and Weinhouse (1953), with rat liver mitochondria.

DBI Influence on the Inhibition of Gluconeogenesis by Ethanol. In Figure 5 is shown that in the presence of 0.08 mM DBI and 10 mM ethanol glucose formation from alanine was strongly decreased. Also, ethanol depressed the endogenous gluconeogenesis completely. Under these conditions the concentrations of lactate, malate, and glycerol 1-phosphate (solid line in Figure 6a) were elevated more than 300% according to the marked rise of the β-hydroxybutyrate/acetoacetate ratio (Table II). In the presence of ethanol alone (without DBI) the increase of these metabolites was much less (dotted line in Figure 6a). The 3-phosphoglycerate level which usually responds to changes of free NADH (as demonstrated in Figure 3a,b) was not significantly altered by DBI in the presence of ethanol.

We previously reported (Haeckel and Haeckel, 1968) that DBI caused a crossover phenomenon between 3-phosphoglycerate and 3-phosphoglyceraldehyde if fructose was used as substrate for gluconeogenesis. In Figure 6b is shown that the 3-phosphoglycerate concentration is also elevated with alanine in the absence of ethanol. This effect of the guanidine compound explains why the 3-phosphoglycerate concentration is not further decreased by DBI in the presence of ethanol despite the marked rise in reducing equivalents.

TABLE IV: Effect of Ethanol, Acetaldehyde, and Acetate on Oxygen Uptake by Liver Mitochondria, Obtained from Guinea Pigs Fasted for 24 hr.

	$Q_N^{O_2}$	P/O
Control[a]	146[c]	3.6
Ethanol[b]	155	3.4
Acetate	244	3.2
Acetaldehyde	4	0
Ethanol, Acetate, Acetaldehyde	0	0

[a] All samples were incubated with 10 mM pyruvate as described under Methods. The final protein concentration was 10.6 mg/ml. [b] Ethanol, acetate, and acetaldehyde were added in concentrations of 10 mM. [c] Each number represents a mean value of two determinations.

Discussion

No data on the rate of gluconeogenesis in the perfused guinea pig liver are available in the literature. In

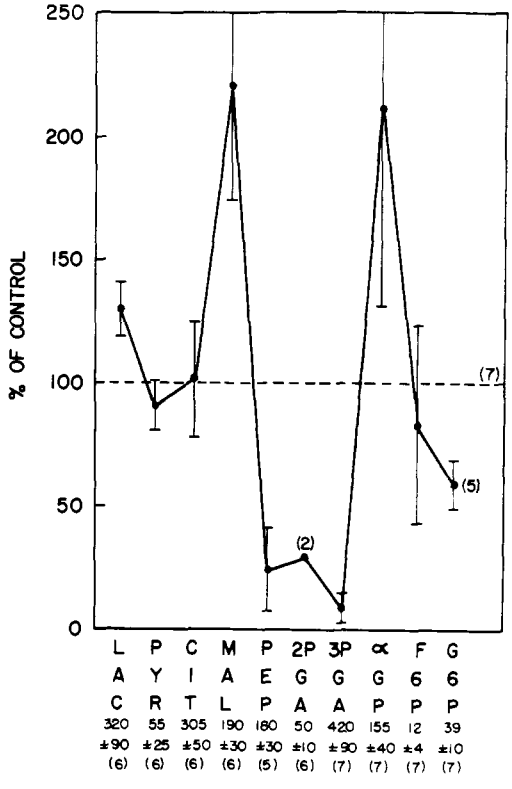

FIGURE 4: Influence of ethanol on metabolite formation in the presence of alanine. For further explanation of symbols, see the legend to Figure 3.

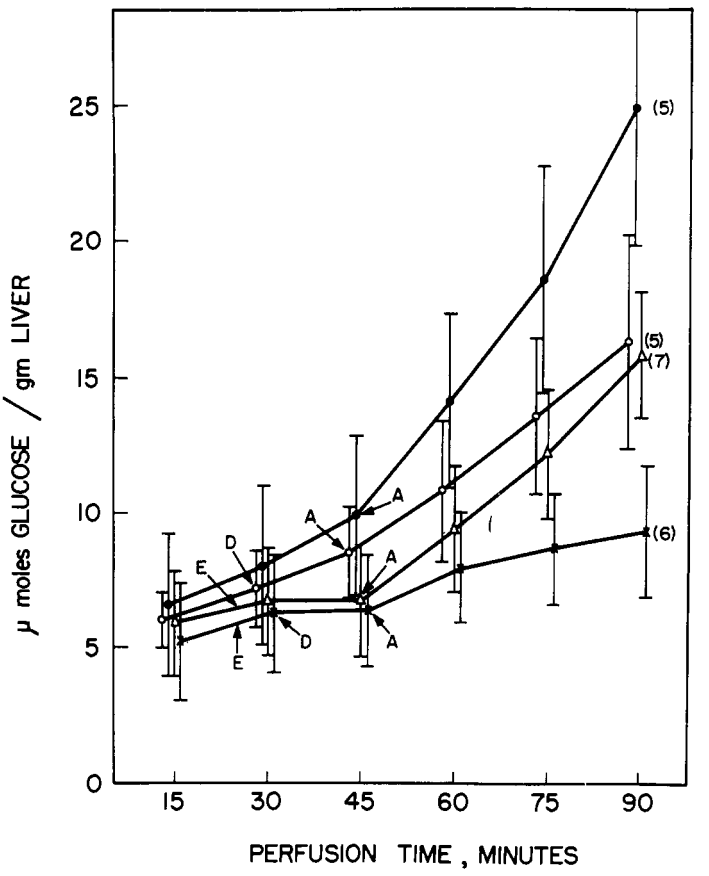

FIGURE 5: Influence of DBI and ethanol on glucose production by the perfused guinea pig liver in the presence of alanine. E indicates the addition of 10 mM ethanol. D means 0.08 mM DBI and A 10 mM alanine. See the legend to Figure 1 for further explanation of symbols.

Table V we compare the amount of glucose formed from the substrates used in the present studies in livers from starved guinea pigs and rats (Ross *et al.*, 1967b). The rate of endogenous gluconeogenesis was subtracted. No significant differences were observed when pyruvate, lactate, or alanine was used. However, fructose was converted about 2.5 times faster in the rat liver.

In our present studies we used only a single dose of ethanol (10 mM), which is high enough to saturate the alcohol dehydrogenase. The K_m for the liver enzyme is approximately 0.54 mM (Sund and Theorell, 1963). With rat liver perfusions Kreisberg (1967) observed no significant difference when 10 mM ethanol was administered as a single dose or when this concentration was kept at the same level by infusion.

In our experiments 10 mM ethanol affected the rate of glucose formation from alanine, lactate, and pyruvate. The most dramatic changes were found in the 3-phosphoglycerate level. Ross *et al.* (1967a) reported that the rate of gluconeogenesis from pyruvate at 10 mM was only 70% of that at 5 mM and suggested that this inhibition may be due to a lack of NADH for the 3-phosphoglyceraldehyde dehydrogenase. We have now presented direct evidence that at high concentrations of pyruvate the conversion of 3-phosphoglycerate into 3-phosphoglyceraldehyde is the rate-limiting step. The addition of ethanol supplies the cytoplasm with enough free NADH to prevent an accumulation of 3-phosphoglycerate. With lactate and alanine, gluconeogenesis is decreased by ethanol despite the stimulation of this step. In the isolated perfused rat liver 10 mM ethanol administered after the addition of alanine stimulates (Williamson, 1968) or at least does not suppress (Kreisberg, 1967) the conversion of alanine into glucose. The increase of the hepatic concentrations of lactate, malate, and glycerol 1-phosphate showed that the dehydrogenase reactions coupled with these metabolites are shifted toward a more reduced state by ethanol. Therefore, the concentration of pyruvate and presumably oxalacetate and dihydroxyacetone phosphate are lowered; this could lead to a decreased flow rate of the gluconeogenic pathway during ethanol oxidation in

TABLE V: The Rate of Glucose Formation in Perfused Livers from Fasted Rats and Guinea Pigs.

Substrate	Rat[a]	Guinea Pig
Alanine	0.52 (6)[b]	0.56 (5)[c]
Pyruvate	0.88 (5)	0.83 (7)[c]
Lactate	0.92 (12)	1.16 (6)[c]
Fructose	2.54 (4)	1.06 (6)[c]

[a] These data were obtained from Ross *et al.* (1967b). [b] All rates of glucose formation are expressed in micromoles per minute per gram of liver. The number of perfusions which contribute to the mean values is given in parentheses. [c] Calculated from the rate of gluconeogenesis during the last 5 min of the perfusion experiments.

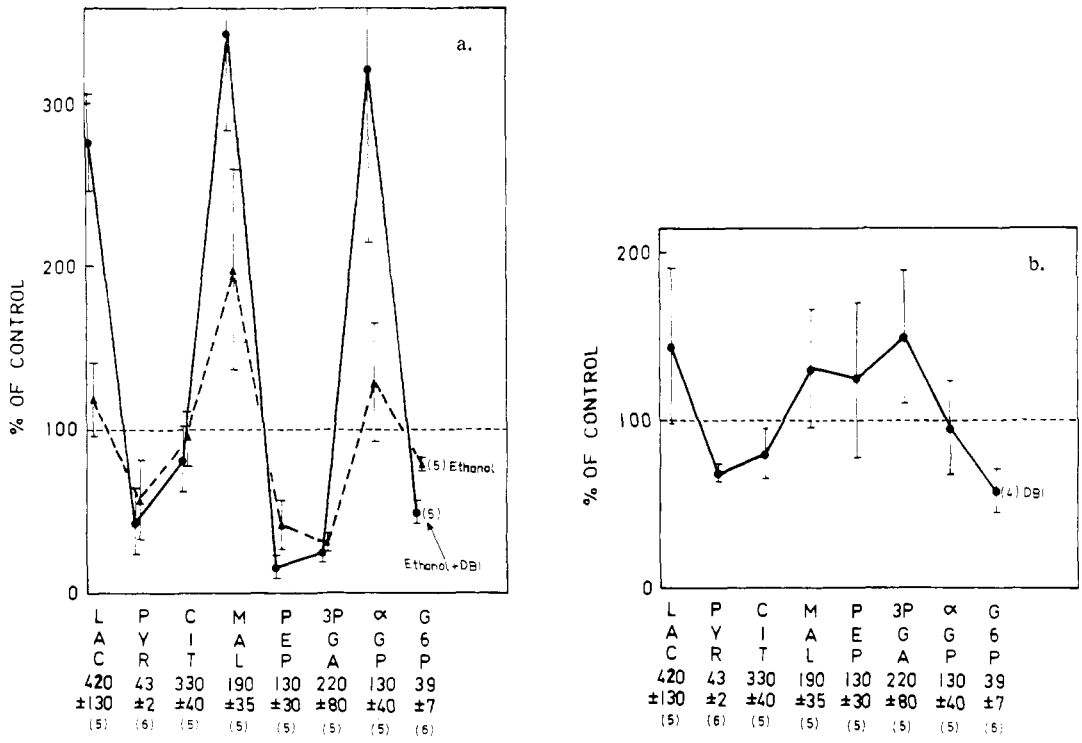

FIGURE 6: Influence of DBI. (a) On gluconeogenic metabolite formation in the presence of ethanol and alanine. Alanine, ethanol, and DBI were added as shown in Figure 5. The control values were obtained in the absence of DBI and ethanol. See Figure 3a for further explanation of symbols. (b) In the presence of alanine alone (without ethanol). DBI and alanine were added to the perfusion medium as shown in Figure 5. See Figure 3a for further explanation of symbols.

the presence of alanine or lactate. This effect of ethanol seemed to be enhanced by 0.08 mM DBI. The drop in the ATP/ADP ratio (Table III) indicated an inhibition of cell respiration by the guanidine compound resulting in a marked accumulation of reducing equivalents in the presence of ethanol. Under these conditions the citrate concentration was lowered (Figure 6). This could lead to an increase of the phosphofructokinase activity according to Williamson (1968). However, the concentrations of fructose diphosphate and of the triose phosphates (Table VI) were lowered rather than increased by DBI in the presence of ethanol. In conclusion, we assume that in the perfused guinea pig liver, the inhibition of gluconeogenesis by ethanol is correlated to an increase of reducing equivalents in the cytosol and not to possible changes of the Krebs cycle activity under the conditions used.

The increase of α-glycerophosphate may stimulate triglyceride synthesis and play a key role in the production of fatty liver by ethanol (Sandler and Freinkel, 1966). Nikkilä and Ojala (1963), Forsander *et al.* (1958), and Baron *et al.* (1967) also reported a rise in the α-glycerophosphate content of rat's liver under influence of ethanol.

In rat liver perfusions ethanol lowers the lactate production (Kreisberg, 1967), whereas in human beings an increase in blood lactate is reported (Mendeloff, 1954). Under all conditions used during these studies we observed an elevation of the hepatic lactate level. Therefore, the results obtained from studies with guinea pig liver may be more suitably compared with the metabolism in the human liver. The concentrations of ketone bodies were increased only shortly after the addition of alanine. Menahan *et al.* (1968) have shown that in the perfused rat liver, acetate stimulated ketogenesis in the absence of other substrates. However, the addition of lactate completely suppressed this effect of acetate. In analogy to this observation we assume that the oxidation of ethanol also leads to an increased ketogenesis in the perfused guinea pig liver, presumably due to a lack of endogenous oxalacetate. If pyruvate is supplied more acetyl-CoA generated from acetate or ethanol can be introduced into the Krebs cycle. Therefore, no elevation of the ketone body concentration was observed 15

TABLE VI: Hepatic Concentrations of the Triose Phosphates in the Presence of Ethanol.

DBI (0.08 mM)	−	+
DAP[a]	20 ± 5 (4)	14 ± 4 (4)
GAP	12 ± 3 (4)	10 ± 4 (4)
FDP	10 ± 4 (4)	3 ± 1 (4)

[a] The concentrations were determined in liver samples taken after 90-min perfusion (substrate, alanine) and are given in nanomoles per gram wet weight with standard deviations and the number of the contributing values.

min after the addition of pyruvate or lactate to the medium. With alanine, apparently a longer time period is required to remove the accumulated ketone bodies.

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