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STUDIES WITH DL-ALANINE-2-^{14}C OF INTERACTION OF ETHANOL METABOLISM WITH PATHWAYS OF HEPATIC GLUCONEOGENESIS IN THE RAT*

-by-

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DISCLAIMER

Running Title

ETHANOL EFFECT ON PATHWAYS OF GLUCONEOGENESIS

Abstract

Studies with DL-alanine-2-^{14}C of interaction of ethanol metabolism with pathways of hepatic gluconeogenesis in the rat. S. Joubert and W.W. Shreeve. Biochem. J. (Brookhaven National Laboratory, Upton, L.I., New York). It was postulated that metabolism of ethanol loads would change the distribution of ^{14}C in hepatic glutamic acid labeled from DL-alanine-2-^{14}C if changes in redox potential of the $NAD^+/NADH$ system, occasioned by ethanol load, inhibit the oxidation of malic acid and thus decrease activity of the gluconeogenic pathway via dicarboxylic acids. However, ethanol metabolism enhanced rather than decreased the labeling of C-2 and C-3 of glutamate (indicative of CO_2 fixation with pyruvate) relative to the labeling of C-5 (oxidative decarboxylation of pyruvate to acetyl CoA), thus suggesting that oxidation of malate was uninhibited relative to oxidative decarboxylation of pyruvate.

Recognition of alcoholic hypoglycemia in man as a clinical entity (Neame and Joubert, 1961), (Field, Williams and Mortimer, 1963), (Freinkel, Singer, Arky, Bleicher, Anderson and Silbert, 1963) has focussed attention on the possible interrelation between metabolism of ethanol and synthesis of glucose. The paradox of both hypo-and hyperglycemic responses to ethanol in experimental animals (Klingman, Haag and Bane, 1958), (Lange, 1960), (Bleicher, Freinkel, Byrne and Seifert, 1964) is unresolved and the mechanism in either case remains obscure. The action of ethanol could be mediated through endocrine factors which exercise homeostatic control over gluconeogenesis, but the possibility of an independent and more direct effect is not excluded.

Administration of ethanol is known to cause a decrease in hepatic NAD^+/NADH ratios (Reboucas and Isselbacher, 1961), (Smith and Newman, 1959). If intracellular NAD^+ is to a certain extent kinetically homogeneous, the operation of other dehydrogenase enzymes which also use NAD^+ may be hampered by the lessened availability of the oxidized form of this coenzyme. One of these enzymes which may function in a direct pathway for gluconeogenesis via dicarboxylic acids is malic acid dehydrogenase. To study the oxidation of malate <u>in vivo</u> during ethanol metabolism, the observations of Freedman and Graff (1958) are pertinent. They showed that the administration of DL-alanine-2-^{14}C to rats will label various carbon positions of hepatic glutamic acids. The distribution of ^{14}C in the molecule of glutamic acid depends theoretically on the relative amounts of pyruvate entering the Krebs cycle by oxidative decarboxylation to acetyl CoA (and subsequent condensation with oxaloacetate) and by reductive carboxylation

to malate followed by oxidation of the latter with NAD^+ as co-factor. Since soluble malic acid dehydrogenase would more likely be affected by competition for NAD^+ by ethanol dehydrogenase than would the mitochondrial pyruvic acid decarboxylase, a change in ^{14}C distribution in glutamic acid could signify selective inhibition of malic acid dehydrogenase. Accordingly, the labeling of glutamic acid derived from liver proteins after administration of DL-alanine-2-^{14}C has been studied in rats as a function of ethanol load.

EXPERIMENTAL

Adult, male Sprague-Dawley rats weighing 270 to 470 gm. were used. Before experimental use the animals were kept on ad libitum diets. The rats were studied in "fed" and "starved" states. By "fed" rat is meant one which has had free access to food up to the time the experiment commenced. "Starved" rats were deprived of food for the periods indicated in Table 1.

Four rats were studied in the fed state. Thirty minutes before receiving a tracer dose of DL-alanine-2-^{14}C (0.1 mc/kg) intraperitoneally, solutions of glucose and/or ethanol were administered by stomach tube in the quantities indicated in Table 1. Immediately after giving the tracer dose the rats were placed in a glass metabolic cage which was swept with a slow stream of CO_2-free air. Expired CO_2 was trapped in NaOH solution (2N). Sixty minutes later the rats were removed from the cage and decapitated. Blood for estimation of glucose was collected in beakers containing dry heparin and the livers were removed within 3 minutes of decapitation.

Three rats were studied in the starved state. They received by intubation, solutions of water or ethanol in the quantities indicated in Table 1. Further procedure was similar to that described for the fed rats, except that in one rat the ^{14}C dose was given 3 hours after administration of ethanol.

Samples of DL-alanine-2-^{14}C were obtained from New England Nuclear Corporation, Boston, Mass. and Research Specialties, Berkeley, California. Chemical and radioisotopic purity was established before use by paper chromatography and autoradiography. Isolation and degradation of hepatic L-glutamic acid was carried out according to the procedure described by Freedman and Graff (1958). Total carbon and its radioactivity was determined by the manometric method of Van Slyke, Steele and Plazin (1951). Samples were counted in the gas phase after transferring carbon as CO_2 to the Bernstein-Ballentine proportional counters (Van Slyke, et al, 1951). Blood glucose was determined by a method using glucose oxidase (Glucostat, Worthington Biochemical Corp., Freehold, New Jersey).

RESULTS

In preliminary experiments it was confirmed that administration of ethanol to fed and starved rats causes a mild hyperglycemic response (Lange, 1960). In starved animals the hyperglycemia is more sustained. The response is unrelated to the dose and is non-specific in the sense that substances other than ethanol will cause hyperglycemia (Lange, 1960). This finding is not prejudicial to the current studies since CO_2 fixation is not the only pathway of new glucose synthesis or augmentation of blood glucose. If ethanol acts on malic acid dehydrogenase in the manner

postulated, it could be manifest irrespective of hyperglycemia.

The production of respiratory CO_2 and the percentage of administered ^{14}C appearing in the expired air during the first hour are given in Table 1. Nutritional state of the experimental animals is clearly the dominant factor affecting these parameters. If ethanol has any effect, the present experiments are insufficient to demonstrate any differences.

Blood glucose concentrations at the time of decapitation (Table 1) indicate the degree of hyperglycemia present 90 minutes after ethanol administration. It was confirmed in preliminary experiments that the peak hyperglycemia was 30 minutes after ethanol was given (Lange, 1960). The fed rat which received ethanol and glucose clearly demonstrates the hyperglycemic effect as compared with the fed rat which had glucose only.

The distribution of ^{14}C in the glutamic acid of liver is given in Table 1. As previously reported (Freedman and Graff, 1958) extreme differences are seen between the fed rat which received glucose and the starved rat which received water. The results in the fed rats receiving ethanol suggested that ^{14}C enrichment of C-5 (derived from oxidative decarboxylation of pyruvate-2-^{14}C) is decreased, since other studies with fed rats show on the average higher amounts of activity in this position (Freedman and Graff, 1958) (Koeppe, Mourkides and Hill, 1959). To test this observation a rat was given both glucose and ethanol with the finding that ^{14}C in C-5 is decreased by about 50% compared with the fed rat receiving glucose alone (Table 1).

Table 1 also shows the calculated randomization between C-2 and C-3 as well as between C-4 and C-5 of glutamate (Shreeve, 1952). These calculations add significant information about metabolic pathways and pool

mixing (vide infra).

The results in summary indicate that ethanol did affect the labeling pattern of glutamate by decreasing the enrichment of ^{14}C in C-5 relative to C-2 and C-3 and did not affect significantly the randomization between C-2 and C-3 or C-4 and C-5.

DISCUSSION

It was earlier proposed that a study of the distribution of ^{14}C in hepatic L-glutamic acid, labeled by DL-alanine-2-^{14}C, could indicate whether oxidation of malic acid was inhibited. This argument assumes that glutamic acid is labeled by a pathway (dicarboxylic acid "shuttle") in which malic acid is an obligatory intermediate (Fig. 1). Utter (1963) has recently re-examined the subject of CO_2 fixation to pyruvate in the light of new evidence. Evidently in some species the possibility of an "abbreviated shuttle" (reaction E of Fig. 1) which does not involve "malic enzyme" (reaction A) or malic acid dehydrogenase (reaction C) should be entertained. However, the intracellular localization of the enzymes concerned in the operation of the full "shuttle" (Fig. 1) in the rat are such that the full "shuttle" is still considered to be a likely pathway of gluconeogenesis in this species. Even if reaction E of Fig. 1 is predominant, an excess of NADH could divert oxaloacetate to malate and thus diminish gluconeogenesis and the net amount of ^{14}C appearing in C-2 and C-3 of glutamate.

Since the activities of carbons 2 and 3 relative to carbon 5 of glutamic acid are increased rather than decreased after ethanol load, the present findings are clearly not consistent with an inhibition of

oxidation of malic acid. Carbon 5 is labeled by oxidative decarboxylation of pyruvate-2-^{14}C to acetyl-1^{14}C CoA, and since acetyl CoA is considered a metabolic product of ethanol (Lundquist, Fugmann, Klanig and Rasmussen, 1959), pool dilution appears to be the likely explanation for lower activity in C-5 after ethanol load.

The randomization between C-2 and C-3 of 80-90% in the present study agrees with that found by Koeppe et al (1959) upon analysis of glutamic acid after administration of pyruvate-2-^{14}C and is somewhat higher than the randomization seen by Freedman and Graff (1958) using DL-alanine-2-^{14}C. The randomization of ^{14}C between C-5 and C-6 of glucose after administration of lactate-2-^{14}C to the intact rat (Friedman, Levin and Weinhouse, 1956) and in other studies (Shreeve and De Meutter, 1964) is like that found in glutamic acid. This would suggest a similar route of metabolism for labeling of both glutamic acid and glucose. However, incomplete randomization in glucose carbons has been taken to indicate the operation of pyruvic kinase (reaction F of Fig. 1) rather than incomplete equilibration of malate or oxaloacetate with the symmetrical dicarboxylic acid, fumarate, (reactions B and C of Fig. 1), because both DL-malate-3-^{14}C (Hoberman and D'Amado, 1962) and DL-aspartate-3-^{14}C (Bloom and Foster, 1962) are converted to glucose with equal labeling in C-5 and C-6. Thus, to explain the incomplete randomization in glutamic acid it would appear necessary to suppose that phosphoenolpyruvate is being converted to oxaloacetate via reaction D. This is opposite to the direction which this reaction is assumed to proceed <u>in vivo</u> for the purpose of gluconeogenesis.

The fact that ethanol load (and presumably excessive conversion of NAD^+ to NADH) did not change the degree of randomization between C-2 and

C-3 constitutes further supportive evidence that the relative activities of the reactions contained in Fig. 1 remained the same and their normal functioning in the process of gluconeogenesis was undisturbed.

A finding of definite interest is the randomization between C-4 and C-5 of glutamate. Carbon 5 becomes labeled as a result of oxidative decarboxylation of pyruvate-2-^{14}C and carbon 4 likewise from pyruvate-3-^{14}C. Randomization between these two carbons therefore, also constitutes a measure of the equilibration of pyruvate with symmetrical intermediates of the Krebs cycle. Since the randomization between C-4 and C-5 is much less than that between C-2 and C-3 of glutamate, at least in the fed animals, it seems that there was little reformation of pyruvate from dicarboxylic acids via reactions A, E or D and F of Fig. 1. This confirms a previous impression from randomization in acetyl groups compared with glucose (Shreeve, 1952) after administration of pyruvate-2-^{14}C.

Whereas in the fed rats only 1 or 2 in every 10 molecules of pyruvate entering the Krebs cycle via acetyl CoA had equilibrated with symmetrical dicarboxylic acids, 1 in 2 molecules were randomized in 2 out of 3 of the starved animals. Since randomization between C-2 and C-3 of glutamate was essentially unaffected by the nutritional state, a possible conclusion is that under starved conditions phosphoenolpyruvate formed from oxaloacetate equilibrates more rapidly with free pyruvate.

Since the present findings render unlikely the possibility that oxidation of ethanol is coupled to the reduction of oxaloacetate, current studies by the authors with ethanol-1-^{3}H are directed to investigation of a possible interaction of ethanol oxidation with the reduction of other substrates thereby affecting gluconeogenic processes.

SUMMARY

1. It was postulated that metabolism of ethanol loads may interfere with gluconeogenesis via the dicarboxylic acid pathway by inhibiting the oxidation of malic acid because of substrate competition for NAD^+. To gain a measure of the oxidation of malate in the liver *in vivo* the distribution of ^{14}C in hepatic glutamic acid, labeled by DL-alanine-2-^{14}C during ethanol metabolism, was studied in fed and starved rats.

2. It was found that ethanol metabolism enhanced rather than decreased the amount of pyruvate entering the Krebs cycle through CO_2 fixation to dicarboxylic acids (labeling C-2 and C-3 of glutamate) relative to the amount entering as acetyl CoA (labeling primarily C-5 of glutamate), so it is tentatively concluded that changes in redox potential of the NAD^+/NADH system, occasioned by ethanol load, do not affect the intrahepatic oxidation of malate.

3. Randomization of ^{14}C between C-2 and C-3 of glutamate was unaffected by ethanol load or by starvation and randomization between C-4 and C-5 was not changed by ethanol but increased by starvation. Implications of randomization for alternate pathways of pyruvate metabolism and equilibration of substrate pools are discussed.

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FIGURE I

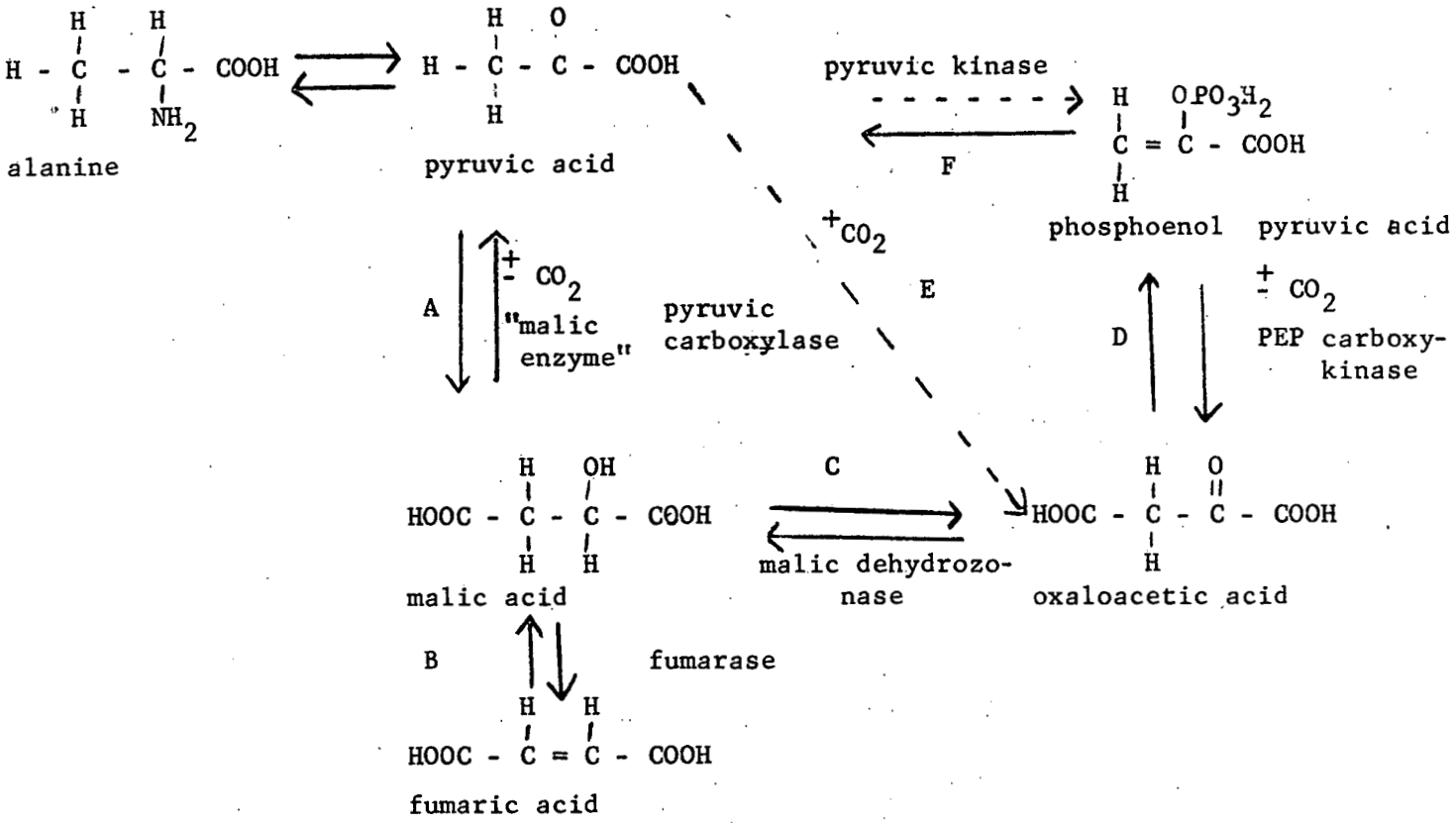
alanine
pyruvic acid
pyruvic kinase
F
phosphoenol pyruvic acid
+CO2
E
pyruvic carboxylase
A
± CO2
"malic enzyme"
D
± CO2
PEP carboxy-kinase
C
malic dehydrozo-nase
malic acid
oxaloacetic acid
B
fumarase
fumaric acid

Table 1. Experimental Conditions and Findings after Intraperitoneal Injection of DL-Alanine-2-^{14}C in Rats

Nutritional State of Rats	Experimental Conditions	Total Carbon in Expired Air mg/Kg/hr.	% of Dose of ^{14}C in Expired Air	Concentration of Blood Glucose mg/100 ml	Distribution of ^{14}C in Hepatic L-Glutamic Acid						% Randomization* Between C-2 and C-3 of L-Glutamic Acid	% Randomization* Between C-4 and C-5 of L-Glutamic Acid
					C-1	C-2	C-3	C-4	C-5	Total**		
FED	5.0 ml 50% (v/v) glucose 30 min. before DL-alanine-2-^{14}C	500	18.6	120	17.3	17.2	23.7	2.0	38.8	99.0	84	10
	5.0 ml 20% (v/v) ethanol 30 min. before DL-alanine-2-^{14}C	418	18.0	96	10.2	34.5	44.8	0.8	9.2	99.5	88	16
	6.0 ml 50% (v/v) ethanol 30 min. before DL-alanine-2-^{14}C	400	13.8	98	12.5	29.0	41.7	0.9	15.1	99.2	82	11
	5.0 ml 50% (w/v) glucose containing 1.0 ml ethanol 30 min. before DL-alanine-2-^{14}C	600	16.4	210	16.1	27.0	29.0	2.0	22.9	97.0	96	17
STARVED	5.0 ml water 30 min. before DL-alanine-2-^{14}C (starved 64 hrs.)	334	7.3	78	7.9	40.7	46.0	1.0	3.2	98.8	93	48
	5.0 ml 20% (v/v) ethanol 30 min. before DL-alanine-2-^{14}C (starved 48 hrs.)	308	8.1	93	6.0	36.6	52.0	0.2	2.2	97.0	82	17
	5.0 ml 20% (v/v) ethanol 180 min. before DL-alanine-2-^{14}C (starved 48 hrs.)	385	11.0	89	6.8	40.0	50.0	0.5	1.6	98.9	89	48

$$\% \text{ Randomization} = \frac{2a}{a+b} \times 100,$$ where a is specific activity of C-2 or C-4 of L-glutamic acid and b is specific activity of C-3 or C-5 of L-glutamic acid (14)

** Sum of radioactivities of individual carbons relative to 100 by total combustion of aliquots of glutamic acid.