Influence of L-Tryptophan and Its Metabolites on Gluconeogenesis in the Isolated, Perfused Liver*

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ABSTRACT: The addition of 2.4 mM tryptophan to the medium perfusing isolated rat livers inhibited glucose production from added alanine. Such livers retained the capacity for converting fructose to glucose. The inhibition of gluconeogenesis was accompanied by elevations in the liver content of aspartate, malate, and oxalacetate, and by decreases in the content of phosphoenolpyruvate, 2-phosphoglycerate, and 3-phosphoglycerate. Paradoxically, tryptophan was found to increase the assayable activity of phosphoenolpyruvate carboxykinase. These results confirm previous findings concerning the effects of tryptophan in intact rats. L-Kynurenine, L-3-hydroxykynurenine, 3-hydroxyanthranilic acid, and quinolinic acid, metabolites in the sequence of reactions from tryptophan to nicotinic acid, were found to duplicate the effects of tryptophan on gluconeogenesis in the perfused liver. Quinolinic acid also increased the assayable activity of phosphoenolpyruvate carboxykinase. Nicotinic acid did not inhibit gluconeogenesis. The hypothesis is offered that tryptophan and certain of its metabolites inhibit gluconeogenesis by being converted to quinolinic acid, a compound whose ferrous coordination complex inhibits the enzyme phosphoenolpyruvate carboxykinase. The addition of 6 mM caprylate to the perfusing medium containing 2.4 mM tryptophan or quinolinic acid greatly enhanced the accumulation of aspartate and malate. This is offered as evidence that at least a part of the stimulating influence of fatty acids on gluconeogenesis is mediated by the enhanced conversion of pyruvate to four-carbon dicarboxylic acids.

The hepatic enzyme, phosphoenolpyruvic carboxykinase (Utter and Kurahashi, 1954), plays a vital role in the synthesis of glucose from dicarboxylic acids. The activity of the enzyme has been found to be influenced by factors known to alter the rate of gluconeogenesis (Shrago *et al.*, 1963; Foster *et al.*, 1966a). Recently, it has been discovered that the administration of L-tryptophan to intact or adrenalectomized rats greatly elevates the PEP[1] carboxykinase activity measured in the 105,000*g* (1 hr) supernatant fraction of liver, although *in vitro* addition of the amino acid has no effect on activity (Foster *et al.*, 1966b). In the rat, L-tryptophan blocks the glycogenic effect of hydrocortisone and prevents glycogenesis from pyruvate, malate, and aspartate, but not from glucose or glycerol (Foster *et al.*, 1966b; Ray *et al.*, 1966). Analyses of livers of rats given L-tryptophan for intermediates of gluconeogenesis disclosed an elevation in the concentrations of lactate, pyruvate, aspartate, malate, and oxalacetate, is association with a decrease in the concentrations of PEP, 2-phosphoglycerate, 3-phosphoglycerate, dihydroxyacetone phosphate, fructose 6-phosphate, and glucose 6-phosphate (Ray *et al.*, 1966). These data are consistent with the *in vivo* inhibition of hepatic PEP carboxykinase in rats given L-tryptophan.

The pattern of hepatic gluconeogenic intermediate concentrations after L-tryptophan administration supports previous findings concerning the role of malate, aspartate, and citrate in gluconeogenesis (Lardy *et al.*, 1965; Walter *et al.*, 1966). A system less complex than the intact rat was required to investigate the mode of action of tryptophan. Since responses could not be obtained in cell-free systems, we investigated the influence of tryptophan on gluconeogenesis in the isolated, perfused rat liver. Tryptophan was found to influence (1) the hepatic concentrations of gluconeogenic intermediates when alanine was used as substrate, (2) the conversion of alanine to glucose, and (3) the activity of PEP carboxykinase. It was found that certain metabolites of tryptophan also inhibit gluconeogenesis in the isolated liver. Data are presented indicating that not tryptophan but one of its major metabolic products is responsible for the three effects described. In addition, it was demonstrated that, in the isolated liver, fatty acid augments the production of gluconeogenic precursors from alanine.

Materials and Methods

Male Sprague–Dawley rats fasted 24 hr were the source of livers used in the perfusion experiments. The perfusate consisted of rat erythrocytes from 75 ml of whole blood, suspended in Krebs-Ringers bicarbonate

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[1] Abbreviations used: PEP, phosphoenolpyruvate; acetyl-CoA, acetyl coenzyme A; DPN^+ and DPNH, oxidized and reduced diphosphopyridine nucleotides; glucose- and fructose-6-P, glucose and fructose 6-phosphates; ITP, inosine triphosphate; ABA, Armour's bovine albumin.

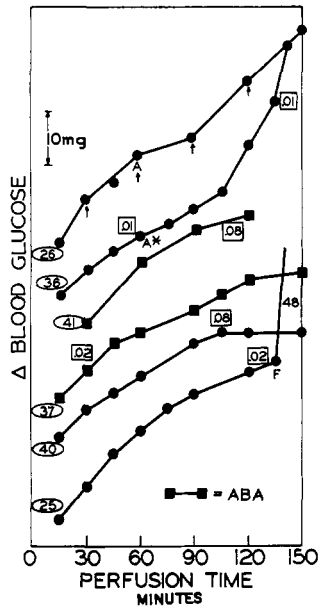

FIGURE 1: Glucose formation with and without added substrate. The circled numbers are initial glucose concentrations in mg/100 ml. The ordinate scale is designated at the top of the figure. The boxed numbers are liver glycogen content measured as g of glucose/100 g of liver. The number at the far right of certain curves is mg of glucose produced/15 min following the addition of 10 mM fructose at F. A indicates the addition of 20 mM L-alanine to the central reservoir. A* indicates 20 mM alanine containing 25 μc of [U^{14}C]-alanine. Arrows pointing upward indicate that 300–500 mg of liver was excised for enzyme assay. ABA, see Materials and Methods.

solution (Umbreit *et al.*, 1964) and 3% (w/v) bovine serum albumin. The albumin was fatty acid poor (Nutritional Biochemicals Corp., Cleveland, Ohio) except in some designated experiments where Armour's bovine albumin (ABA) was used. The type of albumin used did not alter significantly any of the parameters measured in this study. In all cases the total volume of the perfusate was 100 ml.

The perfusion method was that of Brauer *et al.* (1951). The rats were anesthetized with ether and an incision was made in the abdomen allowing maximum exposure. The bile duct, portal vein, and inferior vena cava were cannulated in that order. Heparin was used as anticoagulant. The liver was excised and connected to the perfusion apparatus. The liver rests, diaphragmatic surface downward, on a siliconized rubber dam in a petri dish. Arterial blood enters through the portal cannula and venous blood leaves through the vena cava cannula. In the outflow a glass collecting bulb serves to prevent siphoning of perfusate through the organ (Flock and Owen, 1965). Oxygenation is carried out throughout the perfusion by means of the mixture of 95% oxygen and 5% carbon dioxide under 1 atm of pressure; the gas is humidified by first passing it through isotonic saline. The temperature of the perfusion chamber is 37°.

Blood flow through the liver was maintained at about 2.0 ml/min per g wet wt although flow rates of up to 3.6 ml/min per g of liver were possible in our system. Glucose production is accepted as the major criterion of viability. In experiments where gluconeogenesis from three-carbon compounds was blocked, viability was tested near the completion of the experiment by determining blood glucose levels after addition of fructose or galactose to the perfusing medium. Additional accepted criteria of viability in order of importance are blood flow, appearance of the liver, and bile production. Blood glucose was determined by the glucostat method (Worthington Biochemical Corp.) on 0.2-ml aliquots of perfusate drawn from the central reservoir.

The concentrations of substrates, tryptophan, and of its metabolites are given in the appropriate figure legends. All materials were added to the central reservoir in 2- or 5-ml volumes. When the larger volume was required the appropriate correction for dilution was made in calculating glucose concentration.

Liver specimens (about 0.8 g each) were excised at appropriate times, immersed in 6% (v/v) perchloric acid at −5°, and homogenized within 5–8 sec of removal. Pestle, homogenizer, and its perchloric acid content were preweighed and reweighed to determine the wet weight of liver. The liver homogenate was centrifuged at 5° (5000*g*) for 20 min; the supernatant fraction was collected and the precipitated protein was washed with additional perchloric acid and centrifuged again. The combined supernatant fractions were adjusted to pH 3–4 using potassium carbonate solution. Water was added so that each gram of liver yielded an extract of 8.0 ml. This method of sampling was compared to quick freezing in a tissue press as used in this laboratory (Ray *et al.*, 1966). No significant differences in amounts of metabolites were found and the ratios of lactate-pyruvate and malate–oxalacetate did not indicate an influence of anaerobiosis on DPNH:DPN$^+$.

Pyruvate, PEP, 2-phosphoglyceric acid, 3-phosphoglyceric acid (method of Czok and Eckert), oxalacetate (method of Hohorst and Reim), lactate (method of Hohorst), aspartate (method of Pfleiderer), dihydroxyacetone phosphate and fructose 1,6-diphosphate (method of Bücher and Hohorst), and glucose-6-P and fructose-6-P (method of Hohorst) were determined enzymatically as described in Bergmeyer (1963). Oxalacetate was assayed immediately after neutralizing and pyruvate assays were performed within 24 hr to ensure only minimal losses from instability of these compounds.

Glycogen was isolated (Good *et al.*, 1933) from liver samples of 150–300 mg after digestion of the weighed specimen in 30% KOH. The glycogen was precipitated with 95% ethanol, collected by centrifugation, subsequently washed twice with 60% ethanol, and determined by the anthrone method (Seifter *et al.*, 1950).

PEP carboxykinase was assayed in the supernatant fraction of rat liver homogenized in cold 0.25 M sucrose and centrifuged at 105,000*g* for 1 hr. The assay is based on the measurement of PEP formed during incubation of the enzyme with saturating concentrations of oxalace-

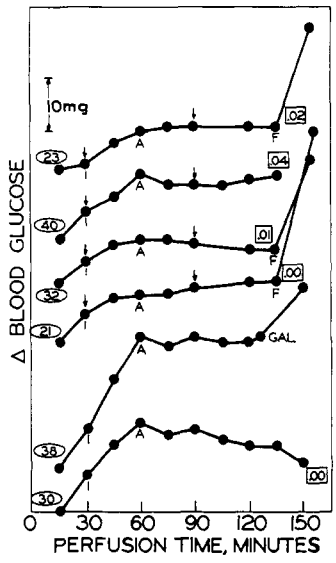

FIGURE 2: Glucose production from alanine by liver perfused with L-tryptophan. The number 1 indicates addition of 2.4 mM tryptophan to the central reservoir. Gal indicates addition of 10 mM galactose. Arrows pointing downward indicate that liver was excised for assay of gluconeogenic intermediates. See Figure 1 for additional abbreviations and symbols.

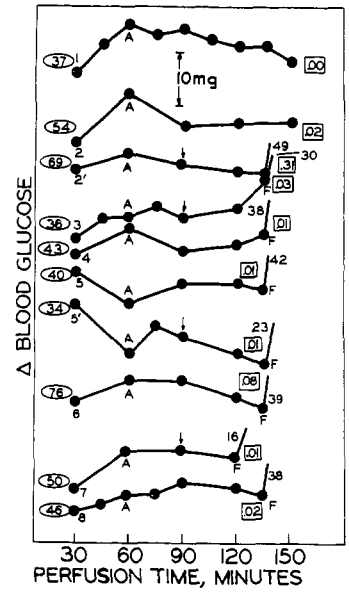

FIGURE 3: Inhibition of gluconeogenesis by metabolites of tryptophan. See Figure 1 legend for abbreviations and symbols. Tryptophan and the various related compounds were added at 30 min, immediately following the removal of a blood sample for measurement of glucose concentration. Key to numbering system: 1, 2.4 mM tryptophan; 2, 4.8 mM DL-kynurenine (ABA); 2′, 2.4 mM L-kynurenine; 3, 2.2 mM L-3-hydroxykynurenine; 4, 2.4 mM quinaldic acid (ABA); 5, 2.4 mM 3-hydroxyanthranilic acid (ABA); 5′, 2.4 mM 3-hydroxyanthranilic acid; 6, 2.4 mM quinolinic acid (ABA); 7, 2.4 mM quinolinic acid; and 8, 4.8 mM quinolic acid.

tate and inosine triphosphate (ITP) (Foster *et al.*, 1966a). PEP was cleaved with mercuric ion and the liberated inorganic phosphate was estimated by the method of Sumner (1944).

Results

Blood Glucose Formation by the Isolated Liver with and without Added Alanine (Figure 1). A rapid net gain in blood glucose occurs during the first 1.5–2 hr of perfusion even when substrate is omitted from the perfusate. This cannot be the result of glycogenolysis because the perfused livers were depleted of glycogen as a result of the 24-hr fast to which all donor animals were subjected. The glucose formation, therefore, must result from gluconeogenesis. This is supported by our and Miller's (1961) finding that the perfused liver forms urea in the absence of added substrate. The prompt conversion of added fructose to glucose at 135 min represents additional evidence that the isolated organ system is capable of rapid metabolic activity. Figure 1 also shows that when alanine was added, the period of glucose production was extended with an increased rate apparent 30 min later. In the radioactivity experiment (A* in Figure 1), the added alanine substrate contained 25 μc of [U-^{14}C]alanine which gave 6.6 $\times$ 10^6 cpm. Eighty minutes later, an aliquot of perfusate was taken and its glucose was precipitated as phenylglucosazone. It was found that the total circulating perfusate glucose contained 1.05 $\times$ 10^6 cpm, demonstrating that the isolated livers utilize added alanine as a carbon source for glucose formation under the conditions described.

Glucose Formation from Alanine by Liver Perfused with 2.4 mM L-Tryptophan (Figure 2). The upper four curves were obtained with livers from which samples were removed at 29 and 90 min. Each sampling removed 5–10% of the total liver mass. In contrast, the two lower curves were derived from livers which remained intact throughout the perfusions. After addition of tryptophan to the perfusate at 30 min, blood glucose continued to increase for 30 min in all six experiments. The addition of alanine at 60 min was followed in all cases by a leveling off in glucose concentration rather than by a hyperglycemic response such as was noted in control livers perfused without tryptophan (Figure 1). When treated for 30 min with 2.4 mM tryptophan, the isolated liver appears to have lost its previous capability for converting alanine to glucose. Such livers, however, retain their capability for the prompt conversion of fructose or galactose to glucose. In other experiments where alanine was not added, complete suppression of glucose accumulation from endogenous carbon sources occurred only after a lag period of 30 min.

The time lag in the inhibition of endogenous gluconeogenesis by tryptophan is consistent with the idea

TABLE I: Influence of Tryptophan Metabolites and of Fatty Acids on Accumulation of Gluconeogenic Intermediates.[d]

Inhibitor	None	L-Trp	L-Kynurenine	L-3-OH-Kynurenine	3-OH-Anthranilate	Quinolinate	Quinolinate	Nicotinate	None	L-Trp	Quinolinate
Column	1	2	3	4	5	6	7	8	9	10	11
Caprylate	0	0	0	0	0	0	0	0	6 mM	6 mM	6 mM
Perfusions	4	4	1	1	1	2[a]	1	2	1	2	1
Lac	0.810	1.47	1.49	2.35	1.31	0.785	0.595	1.76	0.529	1.41	1.34
Pyr	0.197	0.551	0.184	0.795	0.710	0.260	0.232	0.455	0.022	0.070	0.034
Asp	1.06	2.05	3.34	1.26	1.79	3.60	2.93	0.563	5.48	11.88	10.47
Mal	0.094	0.185	0.415	0.171	0.193	0.358	0.287	0.094	0.193	1.23	1.53
OAA	≤0.0019[b]	0.022[c]	—	0.016	0.019	0.028[b]	0.012	—	—	—	≤0.0019
PEP	0.239	0.037	0.060	0.089	0.020	0.071	0.022	0.126	0.125	0.041	0.004
2-PGA	0.059	0.019	0.016	0.030	0.008	0.026	0.006	0.027	0.032	0.007	0.004
3-PGA	0.478	0.090	0.102	0.197	0.078	0.114	0.056	0.260	0.213	0.046	0.078
Lac:Pyr	4.1	2.7	8.1	3.0	1.8	3.0	2.6	3.9	24.0	20.1	39.4

[a] ABA was used. [b] Determination made for one perfusion. [c] Determination made for three perfusions. [d] Alanine (20 mM) was used as substrate in each of the 20 perfusions. Inhibitors and nicotinic acid were added at 30 min after start of perfusion. Alanine was added 30 min later. Assays were done on liver sampled 30 min after addition of substrate. When used, caprylate was added with alanine. All inhibitors were added to give 2.4 mM in the perfusion fluid, except L-3-hydroxykynurenine which was 2.2 mM and nicotinate which was 4.8 mM. PGA, phosphoglyceric acid.

that the actual inhibiting agent is not typtophan but one of its metabolites. If this is so, tryptophan could initially stimulate gluconeogenesis by providing a carbon source in the form of its alanine moiety before the critical concentration of the blocking metabolite is reached.

The Inhibition of Gluconeogenesis from Alanine by Metabolites of Tryptophan (*Figure 3*). A major pathway of tryptophan metabolism in both rat and human livers is initiated by the enzyme tryptophan pyrrolase and terminates with the formation of nicotinic acid. Key intermediates in this sequence of reactions are L-*N*-formylkynurenine, kynurenine, L-3-hydroxykynurenine, 3-hydroxyanthranilic acid, and quinolinic acid (Mahler and Cordes, 1966). The latter compound has been shown to be converted to nicotinic ribonucleotide by condensation with 5-phosphoribosylpyrophosphate under the influence of a beef liver condensing enzyme which has been purified 1500-fold (Gholson *et al.*, 1964). No intermediates in this conversion have been isolated.

Figure 3 shows that L-kynurenine, L-3-hydroxykynurenine, 3-hydroxyanthranilic, and quinolinic acid are effective inhibitors of glucose formation in the perfused liver. Of these, only 3-hydroxyanthranilic acid was capable of turning off glucose formation promptly after its addition to the perfusate as evidenced by a decrease in blood glucose concentration. Doubling the concentration of quinolinic acid failed to stop endogenous gluconeogenesis completely (expt 8, Figure 3). The inhibition of glucose formation from alanine appears to be a specific action of the inhibitors rather than a nonspecific toxic action, for the livers maintain their capability of converting fructose to glucose. The experiment with quinaldate has been replicated with similar results. Only a single experiment was conducted with 3-hydroxykynurenine because of its cost.

From Figure 4 it is evident that 1.2 mM quinolinic acid is only partially effective in preventing glucose formation; lesser amounts were ineffective. In addition, Figure 4 shows that xanthurenic, picolinic, and nicotinic acids do not inhibit glucose formation.

When used at concentrations of 4.8 mM in two additional perfusions not shown graphically, nicotinic acid failed to inhibit glucose production from alanine. Hepatic metabolite data from these experiments are presented in Table I and show that it is not an inhibitor. When xanthurenic acid was perfused at 7.2 mM, it was an effective inhibitor, indicating that adequate cellular penetration did not occur at the 2.4 mM concentration. Picolinic acid failed to inhibit glucose production even when added to the perfusing blood to give a concentration of 7.2 mM.

Influence of Tryptophan, Kynurenine, 3-Hydroxykynurenine, 3-Hydroxyanthranilic Acid, and Quinolinic Acid on Metabolite Formation in the Presence of Added Alanine. Figure 5 depicts the pattern of hepatic metabolite concentrations in isolated perfused livers under the influence of tryptophan. Lactate, pyruvate, aspartate, malate, and oxalacetate accumulated whereas phosphoenolpyruvate, 2-phosphoglycerate, and 3-phosphoglycerate decreased to less than half the control values. It is seen that perfusion with alanine alone leads

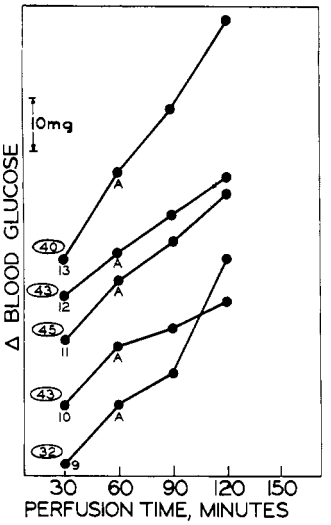

FIGURE 4: Tryptophan metabolites that fail to inhibit glucose production from alanine. See Figure 1 legend for abbreviations. Key to numbering system: 9, 0.49 mM quinolinic acid (ABA); 10, 1.2 mM quinolinic acid (ABA); 11, 2.4 mM nicotinic acid (ABA); 12, 2.4 mM xanthurenic acid (ABA); and 13, 2.4 mM picolinic acid (ABA).

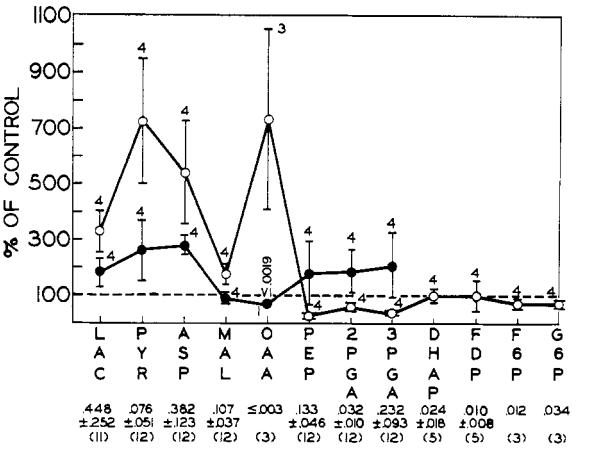

FIGURE 5: Influence of tryptophan on metabolite formation in the presence of added alanine. Control values given in micromoles per gram at the bottom of the figure together with standard deviations and number of experiments (in parentheses), were obtained from lobes removed 30 min after start of perfusion and in the absence of added alanine or tryptophan. The experimental curves are given as per cent of control values. The curve with solid circles represents metabolite concentrations found in lobes taken 30 min after the addition of 20 mM alanine in the absence of tryptophan. The curve with open circles was obtained from lobes taken 30 min after addition of 20 mM alanine but in these experiments 2.4 mM tryptophan had been added 30 min before alanine. The numbers of perfusions contributing to the experimental data are given above the vertical lines that indicate the standard deviations of the means. LAC = lactate, PYR = pyruvate, ASP = aspartate, MAL = malate, OAA = oxalacetate, PEP = phosphoenolpyruvate, 2-PGA = 2-phosphoglycerate, 3-PGA = 3-phosphoglycerate, DHAP = dihydroxyacetone phosphate, FDP = fructose 1,6-diphosphate, F-6-P = fructose 6-phosphate, G-6-P = glucose 6-phosphate.

to increased concentrations of several metabolites but there was no crossover point such as occurred in the presence of tryptophan.

Table I shows the influence in the isolated liver of kynurenine, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, and quinolinic acid (columns 3–7) on hepatic metabolites. In each case the pattern for the concentrations of metabolites is the same as that observed after the addition of tryptophan.

Figure 6 depicts the pattern of metabolite concentrations in liver under the influence of quinolinic acid. After 90 min of perfusion with this substance, the crossover point is still evident.

Reversal of the Quinolinic Acid Block by Addition of $MnCl_2$. Because certain divalent transition metal salts activate PEP carboxykinase (Foster *et al.*, 1967; J. Johnson and H. A. Lardy, unpublished data) experiments were conducted to determine the influence of one of these salts on the inhibition of gluconeogenesis by quinolinic acid. In five control perfusion experiments, 2.4 mM quinolinic acid was added at 30 min and 20 mM alanine at 60 min. Blood glucose concentration failed to increase after substrate addition until 10 mM fructose was added at 120–150 min in the various experiments. In each of three additional experiments, perfusion with quinolinic acid and alanine was carried out as above and 2.4 mM $MnCl_2$ was added at 90, 105, and 126 min, respectively. In these experiments the blood glucose curves were flat from the time alanine was added at 60 min until 15 min after addition of $MnCl_2$ when they increased to the respective rates of 21, 23, and 16 mg/60 min per 10 g of liver. These data show that the quinolinic acid block can be reversed by stoichiometric amounts of Mn^{2+}. Each of these livers retained a normal capability for converting fructose to glucose despite the presence of the high concentration of manganese salt.

Influence of Tryptophan and of Quinolinic Acid on the Assayable Activity of PEP Carboxykinase (Figure 7). In the intact rat hepatic PEP carboxykinase activity increases about 80% in the first 30 min following tryptophan administration (Foster *et al.*, 1966b). Figure 7 demonstrates a definite lag period of 15–30 min for activation of the carboxykinase by tryptophan in perfused livers. In contrast, quinolinic acid brought about a rapid rise in carboxykinase activity during the first 15 min and a slower rise that continues for the duration of the experiments. The assayable PEP carboxykinase activity in control livers, with and without added alanine, was not significantly altered during the experimental period.

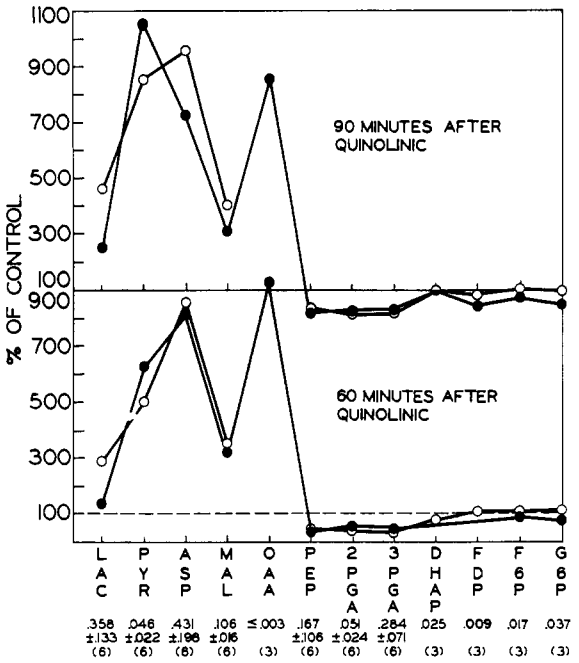

FIGURE 6: Influence of quinolinic acid on metabolite formation in the presence of added alanine. ABA was used for all perfusions. As in Figure 5, control values for metabolites were obtained from lobes removed 30 min after start of perfusion and in the absence of added alanine and quinolinic acid. Quinolinic acid (2.4 mM) was added at 30 min and alanine at 60 min. During each of the two perfusions the livers were sampled at 90 min (lower curves) and at 120 min (upper curves) for assay of gluconeogenic intermediates. The control value for OAA was taken from Figure 5. For abbreviations of intermediates see Figure 5.

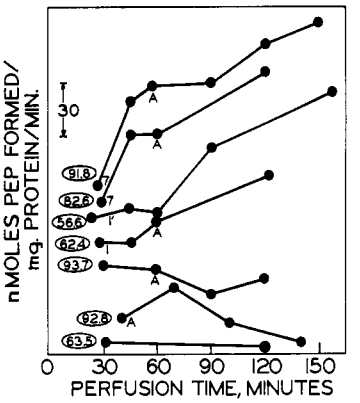

FIGURE 7: Influence of tryptophan and quinolinic acid on the assayable activity of PEPCK of perfused livers. The circled numbers represent enzyme activity in the first liver sample for each perfusion. The lowermost curve was obtained from a perfusion in which neither inhibitor nor alanine was added. The letter A indicates 20 mM alanine. The number 1 indicates the addition of 2.4 mM tryptophan. In expt 1′ whole blood was used as perfusate. This was the only experiment in the entire series in which whole blood was used. The number 7 indicates the addition of 2.4 mM quinolinic acid.

Influence of Fatty Acids. It is well known that the supply of fatty acids to the perfused liver regulates gluconeogenesis (Struck *et al.*, 1965; Williamson *et al.*, 1966). We, therefore, used the block imposed by tryptophan or quinolinate to investigate the site of action of fatty acids. Table II depicts the influence of caprylate on metabolite accumulation in three pairs of complementary liver perfusion experiments using tryptophan as a blocking agent. Each liver served as its own control and the magnitude of change in malate and aspartate concentrations in each experiment is compared to the changes in its complementary experiment. Alanine served as substrate in each perfusion. In expt 1A,B (Table II) the effects of tryptophan on one liver are compared to the effects of both tryptophan and potassium caprylate in the other; alanine was added first in each case. The expected rise in malate and aspartate concentrations occurred 30 and 60 min after tryptophan was added. The combination of tryptophan and caprylate resulted in much greater increases in the concentrations of these metabolites at the corresponding time intervals after tryptophan administration. Note that in this perfusion, malate increased twelvefold and aspartate almost fivefold. In expt 2A,B the effects of sodium caprylate in one liver are compared to the effects of both sodium caprylate and tryptophan in the second; alanine was added simultaneously in each case. Caprylate enhanced malate and especially aspartate accumulation during the first 30 min following alanine addition (expt 2A *vs.* 1A). The ratio of lactate to pyruvate was greatly increased as would be predicted (Lardy, 1965a,b) for a system where caprylate supplies reducing equivalents for malate formation inside the mitochondrion and malate transfers these to the cytosol in the gluconeogenic pathway (Lardy *et al.*, 1965; Walter *et al.*, 1966). In the second 30-min period, as the caprylate was utilized and as malate and aspartate were converted to carbohydrate, the concentrations of these four-carbon acids and the ratio of lactate to pyruvate decreased. When both tryptophan and caprylate were added (2B), malate and aspartate continued to increase in the second 30 min as a result of the presence of tryptophan.

In the last pair of perfusions (3A,B) tryptophan was added 30 min before the substrate to establish the inhibition of gluconeogenesis and the effects of alanine and saline in one liver were compared with the effects of alanine and sodium caprylate on the second liver. Again tryptophan without caprylate led to the expected increases in malate and aspartate. Caprylate after tryptophan in the last experiment, however, resulted in the greatest increases in these intermediates seen in the entire series of six perfusions. Each of the three pairs of experiments differed in design from the other two pairs in the fact that alanine, caprylate, and tryptophan were added to the central reservoir in different sequence.

TABLE II: The Effects of Tryptophan and Caprylate on the Levels of Dicarboxylic Acids in Perfused Liver.

Expt	Additions	Time of Addn[a] (min)	Time of Liver Sample Taken[a] (min)	μmoles/g of Liver: Malate	Aspartate	Lactate	Lactate:Pyruvate	Blood Sugar[b] (mg/100 ml)	Glycogen (%)
1A	Alanine	40	70	0.07	1.07	0.60	2.9	37	0.01
	Tryptophan	72	102	0.20	2.33	0.79	4.4	48	
			132	0.35	4.23	1.22	6.6	51	0.004
1B	Alanine	41	71	0.12	1.09	0.71	6.3	74	0.01
			108	0.98	5.57	0.93	33.4	100	
	Tryptophan + K-caprylate	78							
			138	1.43	5.42	1.02	12.0	102	0.01
2A	Alanine + Na-caprylate + saline (5 ml)	40	70	0.19	5.48	0.53	24.3	50[c] 64	0.02
			100	0.13	0.78	0.89	7.4	81	0.05
2B	Alanine + Na-caprylate + tryptophan	40	70	0.20	5.81	0.58	24.3	31[c] 44	0.005
			100	0.35	7.98	0.88	9.2	57	0.005
3A	Tryptophan	31	28	0.09	0.41	0.60	7.5	44	
	Alanine + saline (2 ml)	61	91	0.23	2.93	1.34	4.4	47	0.04
			140					49	
3B	Tryptophan	30	28	0.08	0.47	0.18	4.4	41	
	Alanine + Na-caprylate	62	92	1.33	13.17	1.50	18.4	42	
			136					41	0.09

[a] All time values refer to time of perfusion. Ala = 20 mM, caprylate = 6.0 mM, Trp = 2.4 mM. [b] Blood sugar values were those found at the time liver specimens were taken. [c] Blood sample taken at 38 min.

The main point to be made is that, whatever the sequence, the combination of tryptophan and caprylate invariably led to much greater accumulation of malate and aspartate than either tryptophan or caprylate alone.

Experiment 3B of Table II was duplicated and the combined results of both experiments are included in column 10 of Table I. The results given in column 11 of Table I show that caprylate caused similar effects when the block of gluconeogenesis was imposed by quinolinate instead of tryptophan.

Discussion

Data presented here show that tryptophan inhibits gluconeogenesis in the isolated rat liver perfused with 20 mM alanine. The effect of tryptophan on the concentrations of hepatic metabolites was to establish a crossover point between oxalacetate and PEP, in agreement with previous findings concerning the effects of tryptophan in the rat (Ray *et al.*, 1966). The data from the whole animal experiments and our perfusion experiments led to the conclusion that the activity of PEP carboxykinase is greatly diminished *in vivo* following administration of tryptophan, and that it is this phenomenon which is responsible for the failure of glycogen and glucose production. The possibility has been entertained that tryptophan might inhibit gluconeogenesis by preventing the migration of malate and aspartate from mitochondria into the cytosol where glucose formation takes place. We are able to reject this possibility by our finding that in the perfused liver system blood aspartate concentrations increase as liver aspartate accumulates as a result of the tryptophan block. In the intact rat, blood malate increases strikingly following tryptophan administration (P. D. Ray, unpublished data). Paradoxically, enzyme assays of livers from tryptophan-treated rats (Foster *et al.*, 1966b) disclosed increased *in vitro* activity of PEP carboxykinase, a finding we are able to duplicate in the isolated liver perfused with tryptophan or with quinolinic acid.

In previous experiments (Foster *et al.*, 1966b; D. O. Foster, P. D. Ray, and H. A. Lardy, unpublished data)

it was found that *N*-formyl-L-kynurenine was approximately half as effective as L-tryptophan in elevating PEP carboxykinase activity of fasted rats' livers. L-Kynurenine and 3-hydroxyanthranilic acid were slightly less effective. With intact animals, excretory processes may diminish the effectiveness of some administered compounds and hormonal release in response to toxic compounds may elevate PEP carboxykinase (Shrago *et al.*, 1963) independently of any direct influence of the compound on enzyme activity. For these reasons we examined in perfused livers the influence of compounds derivable from tryptophan.

Quinolinic acid and the precursors from which it is synthesized in liver, inhibit the conversion of alanine to glucose and produce the same pattern of change in gluconeogenic intermediate concentrations found after tryptophan administration. Nicotinic acid, which gives rise to several products of quinolinate metabolism in mammalian liver, does not inhibit PEP carboxykinase activity in the perfused liver (Figure 4, Table I). Failure of inhibition cannot be attributed to ineffective intracellular concentrations, for the rapid uptake of this substance is well known (Goodman and Gilman, 1965). Therefore, quinolinic acid is the last active metabolite in the major sequence of reactions of tryptophan metabolism. It must be asked if L-tryptophan, L-kynurenine, L-3-hydroxykynurenine, and 3-hydroxyanthranilic acid influence PEP carboxykinase activity themselves or if they exert their influence only by giving rise to quinolinic acid.

Because quinolinic acid is radically different in structure from its precursors, it is difficult to see how the latter could directly exert the same unique influence on glucose production and hepatic metabolite concentration. Therefore, we suggest that quinolinic acid is the active agent and that its precursors inhibit gluconeogenesis by giving rise to it.

The blocking agents differ in the time required for their influence on the perfusate glucose concentrations to be demonstrable (Figure 3). The differences relate to (1) varying rates of cellular uptake, (2) contributions to glucose carbon by the inhibitor before PEP carboxykinase has been rendered inactive, and (3) the time required for effective concentrations of inhibitor to be reached. With the exception of 3-hydroxyanthranilic acid, the precursors of quinolinic acid (Figure 3) show a time lag in their inhibition on blood glucose formation as evidenced by a continued rise in glucose concentration during the 30 min after their addition. Although the lag can in part be secondary to delay in hepatic cellular uptake, it is more likely secondary to the time required for the transformation of these metabolites to quinolinic acid in effective concentrations.

3-Hydroxyanthranilic acid was the most effective inhibitor of endogenous gluconeogenesis (Figure 3). This metabolite is rapidly taken up by liver and converted to quinolinic acid (Professor L. M. Henderson, personal communication). Rapid cellular penetration and conversion to high intracellular concentrations of quinolinic acid might explain the prompt inhibition of endogenous gluconeogenesis caused by 3-hydroxyanthranilic acid. However, 3-hydroxyanthranilic acid, at 1 mM, strongly inhibits mitochondrial oxidation and phosphorylation (Quagliariello *et al.*, 1964, 1966) which, together with an enhancement of liver glycolysis (Papa *et al.*, 1962), could also account for its effects on perfusate glucose concentration.

From Figure 3 it is evident that added quinolinic acid does not turn off glucose formation immediately. This is probably the result of its slow uptake by the liver cell. This has been demonstrated using radioactive quinolinate (Professor L. M. Henderson, personal communication). In addition, it has been shown, using an enzyme purified from beef liver, that quinolinate is rapidly converted to nicotinic acid ribonucleotide and that the K_M for quinolinate is low (Gholson *et al.*, 1964). Slow penetration, and conversion to nicotinic acid derivatives, may explain why quinolinic acid is not a quicker acting inhibitor of endogenous gluconeogenesis in the isolated liver.

Xanthurenic acid did not inhibit glucose formation from alanine when used in a concentration of 2.4 mM (Figure 4). However, at 7.2 mM it was found to be an effective inhibitor. Probably the greater concentration was required to get an effective intracellular concentration. Quinaldic acid inhibits gluconeogenesis but it gave a pattern of intermediates different from that obtained after tryptophan. The mechanism of action of this and other inhibitors of gluconeogenesis will be presented elsewhere.

Heterocyclic acids having a carboxyl group in the α position to the ring nitrogen (quinaldic, picolinic, quinolinic, and xanthurenic) form strong complexes with transition metal ions but bind alkaline earths only weakly or not at all (Bailar, 1956). The inhibition of PEP carboxykinase activity *in vivo* by quinolinic acid may involve the combination of this enzyme with ferrous quinolinate complex (J. Johnston and H. A. Lardy, unpublished data). Whereas inhibitory concentrations of ferrous quinolinate complex may be responsible for inhibition of gluconeogenesis *in vivo*, on homogenization and dilution (300-fold) of the liver for assay of PEP carboxykinase, the inhibitor appears to dissociate from the enzyme leaving it in the metal-activated state (Foster *et al.*, 1967) that is approximately twice as active as the nonactivated enzyme. Details of these studies will be presented elsewhere (J. Johnston and H. A. Lardy, unpublished data).

That quinolinate can form metal complexes under the conditions of the perfusion system used is supported by the observation that the block it imposes on glucose formation can be reversed by the addition of stoichiometric amounts of $MnCl_2$ to the perfusing fluid. It has been found that when 2.4 mM $MnCl_2$ was added to perfusate containing 2.4 mM quinolinic acid, the block of gluconeogenesis was overcome. In addition, we were able to overcome the block imposed by 7.2 mM xanthurenic acid by the addition of 7.2 mM $MnCl_2$. At the end of these perfusions, the livers retained their capability for converting fructose to glucose despite the presence of such large and otherwise toxic concentrations of $MnCl_2$. In the 2.4 and 7.2 mM $MnCl_2$ experi-

ments the amounts of Mn added were, respectively, 13 and 39 mg. When contrasted with the fact that 10 g of rat liver contains a total of only 14 μg of manganese (Thiers and Vallee, 1957), these amounts of added Mn are seen to be extremely high. From these experiments it appears that under the perfusing conditions used, quinolinic acid and xanthurenic acid can chelate manganese ion and that the acids afford the isolated liver protection against the toxic action of Mn^{2+} through the mechanism of chelation.

In the liver perfusion studies of Struck *et al.* (1965) and of Williamson *et al.* (1966), the addition of a fatty acid was shown to lead to a greater net gain in glucose in the perfusion medium and it was concluded that fatty acids can stimulate gluconeogenesis. However, Weber *et al.* (1966) have recently reported that caprylate at concentrations in excess of 1 mM inhibited the glycolytic enzymes hexokinase, glucokinase, phosphofructokinase, and pyruvate kinase in rat liver homogenates but did not affect enzymes responsible for gluconeogenesis. These authors proposed that the ability of fatty acids to promote gluconeogenesis may be, at least in part, a consequence of their inhibitory effect on glucose utilization and especially on pyruvate kinase. Our perfusion experiments with tryptophan and quinolinic acid demonstrate that caprylate increases the rate of formation of the gluconeogenic intermediates malate and aspartate, thereby establishing that fatty acids exert an influence on gluconeogenesis in that part of the gluconeogenic pathway prior to phosphoenolpyruvate. These results cannot be explained on the basis of the inhibitory properties of fatty acids on pyruvate kinase or other glycolytic enzymes.

The conversion of alanine to aspartate and malate most likely proceeds *via* pyruvate and oxalacetate. In a previous paper of this series (Walter *et al.*, 1966) it was shown that liver mitochondria contain amounts of pyruvate carboxylase equivalent to that reported for whole liver, indicating that the pyruvate carboxylation, which represents the first step in the conversion of pyruvate to glucose, occurs predominantly within the mitochondria. It was also demonstrated (Lardy *et al.*, 1965; Walter *et al.*, 1966) that the oxalacetate formed within the mitochondria does not diffuse into the cytosol but instead is converted to malate, aspartate, and citrate. These acids leave the mitochondria and can serve as substrates for glucose formation in the cytosol. Fatty acid oxidation results in increased availability of reducing equivalents for the generation of DPNH (Bode and Klingenberg, 1965). This, *via* mitochondrial malic dehydrogenase, promotes the reduction of oxalacetate formed by carboxylation of pyruvate and the malate formed diffuses from the mitochondria to the cytosol. The increased malate formation brought about by the influence of fatty acid oxidation will elevate the DPNH: DPN ratio in the cytosol, for Klingenberg and Bücher (1960) have found this coenzyme couple to be in approximate equilibrium with both the malate–oxalacetate and the lactate–pyruvate couples in this cell compartment. It has been emphasized in several papers from this aboratory (Lardy *et al.*, 1965; Lardy, 1965a,b; Shrago and Lardy, 1966) that malate diffusing from the mitochondria is the source of reducing equivalents in the cytosol for the conversion of 1,3-diphosphoglycerate to glyceraldehyde 3-phosphate. Williamson *et al.* (1966) and recently Krebs *et al.* (1967) have reported supporting evidence.

The addition of caprylate, tryptophan or quinolinic acid, and alanine to the perfusion medium caused a large increase in the ratio of lactate to pyruvate. This effect is in agreement with the work of other groups (Williamson *et al.*, 1966; Löffler *et al.*, 1965) who observed similar but smaller changes in the absence of a blocking agent. The fact that caprylate caused such large increases in malate formation and in the lactate to pyruvate ratio in our perfusion studies strongly supports the proposed pathway through the mitochondria, which has been questioned by others (Henning *et al.*, 1966).

Fatty acids may influence gluconeogenesis by two additional means. It is known that fatty acids inhibit pyruvate oxidation (Walter *et al.*, 1966; Bremer, 1966) thereby making more pyruvate available for carboxylation to oxalacetate. In experiments with mitochondria this is especially apparent with physiological concentrations of pyruvate (P. Walter, unpublished data). Furthermore, increased levels of acetyl-CoA as a result of fatty acid oxidation (Wieland and Weiss, 1963) may lead to a stimulation of pyruvate carboxylase activity (Utter and Keech, 1960). This is supported by the observation that the pyruvate levels were lower in the presence of caprylate in the perfusion medium than in its absence (Table I). The influences of fatty acids on pyruvate sparing, formation of acetyl-CoA, and in supplying reducing equivalents may represent the major mechanisms by which fatty acids increase the synthesis of malate and aspartate from alanine in our perfusion experiments and increase gluconeogenesis in intact animals.

It is important to realize that the concentration of fatty acids used in our experiments is higher than the normal levels of free fatty acid in plasma (Hales and Kennedy, 1964). Lower concentrations have been found to have similar effects in preliminary experiments and further work is contemplated.

Our finding that L-tryptophan inhibits synthesis of glucose from alanine perhaps bears on the recent work of Silverstein *et al.* (1966) concerning the content of tryptophan and its intermediates in the serum and urine of some patients with malignancies. These patients were subject to hypoglycemic episodes; possibly the pathogenesis of these episodes relates, at least in part, to the presence of L-tryptophan in abnormally high concentrations in serum, and its inhibition of glucose production by liver.

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