Inhibition by Phenylpyruvate of Gluconeogenesis in the Isolated Perfused Rat Liver†

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ABSTRACT: In livers perfused with no substrates, phenylalanine increased the rate of glucose production 3- to 4-fold whereas phenylpyruvate, the major α-keto acid produced in phenylketonuria, abolished glucose production in these livers. Gluconeogenesis from pyruvate (2 mM) was reduced by about 30% by phenylalanine (2–5 mM), but this inhibition appeared to be due to the production of phenylpyruvate. Increasing concentrations (0–5 mM) of phenylpyruvate progressively inhibited gluconeogenesis from pyruvate such that more than 75% inhibition was attained at 5 mM phenylpyruvate in the perfusion medium. Phenylpyruvate (2 mM) also inhibited to varying degrees gluconeogenesis from propionate, malate and alanine. Glucose production from glycerol was not affected. In liver homogenates phenylpyruvate decreased the carboxylation and decarboxylation of pyruvate. This effect was shown to markedly contribute to the reduction of gluconeogenesis from pyruvate by the perfused liver. In addition phenylpyruvate also reduced the decarboxylation of [1-^{14}C]α-ketoglutarate to $^{14}CO_2$ suggesting an inhibitory effect on the tricarboxylic acid cycle activity which may account for the phenylpyruvate-induced reduction in glucose production from a variety of substrates.

The primary defect in classical phenylketonuria, an inborn disorder of phenylalanine metabolism, is the absence of hepatic phenylalanine hydroxylase (Jervis, 1953). In untreated phenylketonuric patients, this defect results in elevated plasma levels of phenylalanine and its metabolites, especially phenylpyruvate. The etiology of cerebral retardation in this disorder has been a subject of considerable interest (for review, see Bickel *et al.*, 1971). An inhibition of energy generation *via* glycolysis in brain by phenylalanine and phenylpyruvate has been proposed by Weber *et al.* (1970) as a major biochemical abnormality during development and differentiation in phenylketonuric children. Recently we observed a 2-fold increase in the levels of pyruvate and lactate in the plasma of untreated phenylketonuric children as opposed to normal individuals (Sutnick *et al.*, 1972), suggesting an impairment in pyruvate metabolism in these patients. The isolated perfused liver is a suitable system to study the effect of phenylalanine and phenylpyruvate on pyruvate metabolism in an intact organ since glucose production from pyruvate and a variety of other substrates can be conveniently monitored.

Materials and Methods

Materials. Male, Sprague-Dawley rats weighing 150–200 g were fed Wayne Laboratory blox (Allied Mills, Chicago, Ill.) and water *ad libitum*. $NaH^{14}CO_3$ (specific radioactivity 10–20 Ci/mol) was purchased from New England Nuclear Corp.; sodium [1-^{14}C]pyruvate (specific radioactivity 14.4 Ci/mol) and [1-^{14}C]α-ketoglutarate (specific radioactivity 10–15 Ci/mol) were obtained from Amersham/Searle Corp. L-Phenylalanine was obtained from General Biochemicals Inc., and sodium phenylpyruvate from Sigma Chemical Co. All other chemicals were purchased from readily available sources.

Liver Perfusion. Rats (150–200 g), starved for 24 hr, were anesthesized by intraperitoneal injection (50 mg/kg of body weight) of sodium pentobarbital (Nembutal). Details of the cannulation of the portal vein and the *vena cava* and the isolation of the liver have been described (Arinze *et al.*, 1973; Jomain-Baum *et al.*, 1973). The technique of liver perfusion was the hemoglobin-free perfusion system (Schnitger *et al.*, 1965; Scholz *et al.*, 1966) and employed a rotating disc oxygenator (Scholz, 1968). The perfusion apparatus was used as a flow-through system in which the venous effluent was not recirculated. The perfusion medium was Krebs–Ringer bicarbonate buffer (pH 7.4) which was freshly made each day and oxygenated continuously with a stream of an O_2:CO_2 mixture (95:5 v/v). Oxygen uptake was monitored by an in-line Clark-type oxygen electrode which measured the oxygen tension in the venous effluent. High flow rates (20–28 ml/min) through the liver were maintained and adjusted as needed to ensure adequate oxygen uptake by each liver. The nonrecirculating system was ideally suited for kinetic studies because it allowed unlimited and rapid sampling of the perfusate. Since the perfusion fluid passed through the liver only once, the continuous infusion permitted the maintenance of low and presumably near physiological levels of substrates throughout the perfusion. However, the large volumes of perfusion fluid used precluded the routine use of albumin. This omission did not affect rates of oxygen consumption, substrate utilization and glucose production in livers perfused with or without fatty acids and rates of gluconeogenesis from high (10 mM) concentrations of lactate were comparable to rates from recycling experiments (Ross *et al.*, 1967; Söling *et al.*, 1970) in which 3% albumin was included in the perfusion medium.

About 5-ml aliquots of the perfusate were removed at frequent intervals and analyzed directly for glucose with a Technicon Auto-analyzer using the glucose oxidase–peroxidase method (Hill and Kessler, 1966). In some experiments, the perfusate was also analyzed for lactate (Hohorst, 1965) and phenylpyruvate (Knox and Pitt, 1957). In experiments where livers were rapidly freeze clamped (Wollenberger *et al.*, 1960)

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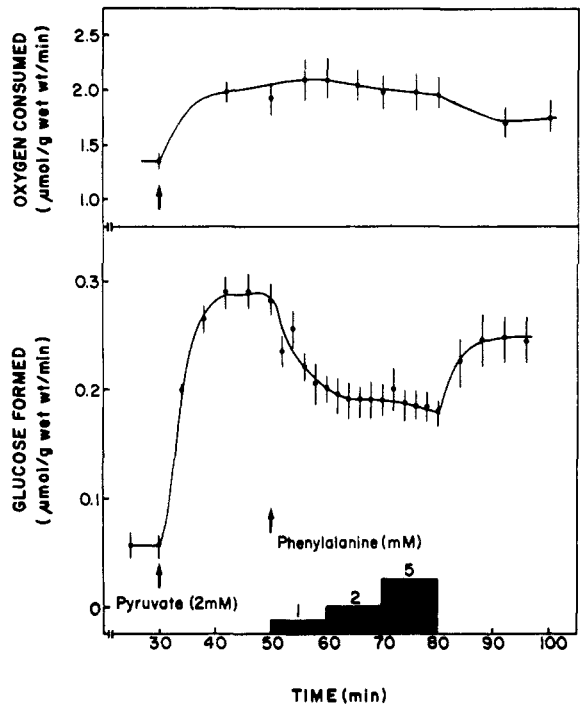

FIGURE 1: Effect of L-phenylalanine on glucose production from pyruvate in the perfused rat liver. Livers from 24-hr fasted rats were preperfused with Krebs–Ringer bicarbonate buffer (pH 7.4) for 30 min. Pyruvate (2 mM) was then introduced and maintained at this concentration for the duration of the experiment. At 50 min L-phenylalanine was infused from a pump to deliver the indicated concentrations at the portal vein input. Points plotted are means ±SEM (vertical bars) for six livers.

TABLE I: Effect of Phenylalanine and Phenylpyruvate on Glucose Production and Oxygen Consumption in Perfused Rat Liver Metabolizing Various Gluconeogenic Substrates.[a]

Substrate	μmol/min per g wet wt	
	Glucose Production	Oxygen Uptake
None (15)	0.032 ± 0.008	1.41 ± 0.09
Phenylalanine (4)	0.137 ± 0.042	2.23 ± 0.11
Phenylpyruvate (4)	0.008 ± 0.003	2.09 ± 0.20
Pyruvate (6)	0.303 ± 0.015	2.00 ± 0.20
+Phenylalanine (6)	0.190 ± 0.017	2.02 ± 0.15
+Phenylpyruvate (6)	0.170 ± 0.012	2.03 ± 0.13
Glycerol (6)	0.430 ± 0.042	2.97 ± 0.12
+Phenylalanine (6)	0.692 ± 0.045	nd
+Phenylpyruvate (6)	0.445 ± 0.047	3.04 ± 0.16
L-Malate (5)	0.073 ± 0.012	1.86 ± 0.13
+Phenylalanine (2)	0.141	2.15
+Phenylpyruvate (3)	0.011 ± 0.002	1.73 ± 0.13
Propionate (3)	0.171 ± 0.034	nd
+Phenylpyruvate (3)	0.059 ± 0.017	nd

[a] Livers from 24-hr starved rats were perfused with substrate (2 mM) for 20 min followed by 2 mM phenylalanine or phenylpyruvate for another 30 min. Glucose production in the absence of added substrate or inhibitor was measured after 30-min perfusion with Krebs–Ringer bicarbonate buffer immediately preceding the addition of substrate or inhibitor. Values given are means ±SEM for the number of animals indicated in parentheses. nd = not determined.

during perfusion, the frozen tissue was pulverized and extracted with perchloric acid as described previously (Arinze *et al.*, 1973) except that the Florisil step was omitted. Established spectrophotometric and enzymatic methods (Bergmeyer, 1963) were used to measure ATP, ADP, AMP, malate, phosphoenolpyruvate, 2-phosphoglycerate, and 3-phosphoglycerate in neutralized extracts. No correction was made for extracellular fluid content in the calculation of tissue metabolite concentrations.

Carboxylation of Pyruvate (Fixation of $H^{14}CO_3^-$) by Liver Homogenates. Rats fed *ad libitum* were killed by cervical dislocation and livers were rapidly removed and transferred into ice-cold buffered sucrose medium (0.3 M sucrose–5 mM triethanolamine buffer, pH 7.4). Homogenates (10% w/v) were prepared in the same sucrose medium. The composition of the reaction mixture and the additions are described in the legend to Figure 4. The fixation of $H^{14}CO_3^-$ in the presence of pyruvate by liver homogenates was measured as described previously (Patel and Hanson, 1970).

Decarboxylation of [1-^{14}C]Pyruvate and [1-^{14}C]α-Ketoglutarate to $^{14}CO_2$ by Liver Homogenates. Liver homogenates were prepared as described above. Incubations were carried out in 25-ml erlenmeyer flasks with rubber serum caps equipped with hanging polyethylene cups. The composition of the reaction mixture, the additions and experimental details are given in the legend to Table II. The reaction was initiated by the addition of liver homogenate. After the appropriate incubation time, 0.4 ml of Hyamine-10× hydroxide was injected into the polyethylene cup through the rubber cap, followed by 0.6 ml of 36% (v/v) perchloric acid into the reaction mixture. The flasks were shaken for an additional 40 min to collect $^{14}CO_2$ and then the content of the cup was transferred into 15 ml of toluene scintillation fluid and the radioactivity was measured.

Results

Effect of Phenylalanine and Phenylpyruvate on Gluconeogenesis in the Perfused Liver. Figure 1 shows the inhibitory effect of phenylalanine on the rate of glucose production from 2 mM pyruvate. The rate of gluconeogenesis fell by about 30% of the initial rate when the phenylalanine concentration in the perfusion medium was 2–5 mM, indicating that saturating levels of the inhibitor had been attained. In these experiments each liver served as its own control. The return of glucose production to near control levels upon termination of the infusion of phenylalanine demonstrates that linear rates of gluconeogenesis were maintained by the nonrecycling system. In livers perfused without substrates, phenylalanine increased the rate of glucose output 3- to 4-fold (Table I), suggesteing that phenylalanine itself may not be the inhibitor in the experiments shown in Figure 1. The increase in O_2 uptake consequent to the addition of phenylalanine is consistent with the metabolism of this amino acid (Meister, 1965). Hepatic phenylalanine aminotransferase (Utena and Saito, 1951; Meister *et al.*, 1956) or aspartate aminotransferase (Miller and Litwack, 1971; Shrawder and Martinez-Carrion, 1972) catalyzes the conversion of phenylalanine to phenylpyruvate. In the experiments of Figure 1 the intracellular concentration of phenylpyruvate was not determined but its concentration in the effluent perfusate was 0.02–0.05 mM when the level of phenylalanine in the perfusion medium was 2–5 mM. When a transaminase inhibitor, aminooxyacetate (Hopper and Segal, 1962), was used at a concentration of 0.2 mM to partially block the conversion of

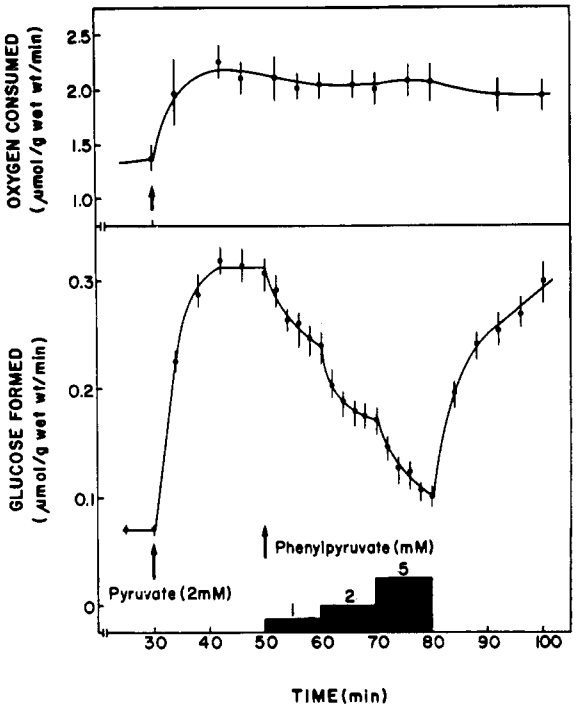

FIGURE 2: Effect of phenylpyruvate on glucose production from pyruvate in the perfused liver. Livers were perfused as described in legend to Figure 1. Points plotted are means ±SEM (vertical bars) for five livers.

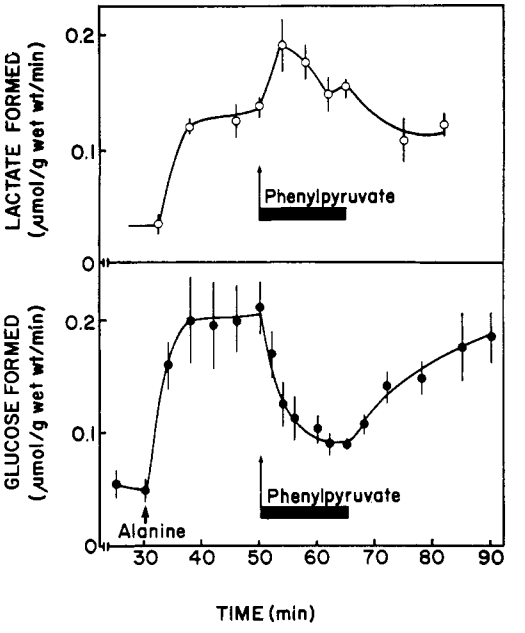

FIGURE 3: Effect of phenylpyruvate on glucose and lactate production from alanine in the perfused liver. Livers were perfused as indicated in the legend to Figure 1. The concentration of L-alanine in the perfusion medium was 2 mM. Beginning at 50 min phenylpyruvate was introduced and maintained at 2 mM by constant infusion for 16 min. Points plotted are means ±SEM (vertical bars) for four experiments.

phenylalanine to phenylpyruvate, the inhibition of glucose production by 5 mM phenylalanine was partially reversed (0.183 ± 0.014 μmol of glucose/min per g in the absence of aminooxyacetate *vs.* 0.221 ± 0.008 in its presence, four experiments). Although this difference is not very impressive, the partial reversal at high phenylalanine concentrations suggests that the inhibition of glucose production in the presence of phenylalanine was perhaps due to the keto acid metabolite of phenylalanine rather than to phenylalanine itself. In parallel experiments, increasing perfusate concentrations of phenylpyruvate progressively inhibited gluconeogenesis such that glucose production was reduced by more than 75% at a phenylpyruvate concentration of 5 mM (Figure 2). Oxygen consumption was not altered and the inhibition of glucose production was completely reversed upon termination of the phenylpyruvate infusion.

In order to locate the site of the observed inhibition, the effect of phenylpyruvate on glucose production from various precursors which enter the gluconeogenic sequence at different stages was tested at equal perfusate concentrations (2 mM) of substrate and inhibitor. Table I shows that gluconeogenesis from glycerol was not affected by phenylpyruvate. With this substrate, there was a significant increase ($P < 0.001$) in glucose production upon the addition of phenylalanine which is explained by the gluconeogenic nature of this amino acid. Glucose production from L-malate was inhibited by about 85% by phenylpyruvate but was stimulated in the presence of phenylalanine. Phenylpyruvate also inhibited gluconeogenesis from propionate by about 65%. The data in Table I suggested that the inhibition occurred below the level of triose phosphates. Attention was therefore directed towards elucidation of the reactions most likely to be affected by the inhibitor. In this regard, inhibition of pyruvate utilization appeared most likely on the basis of previous studies with rat brain mitochondria (Patel, 1972). Therefore, we measured glucose and lactate production from alanine in the perfusate before, during and after the infusion of phenylpyruvate (Figure 3). Upon phenylpyruvate infusion, the rapid decrease in glucose production was accompanied by an immediate rise in lactate production which fell off to the initial values when the phenylpyruvate infusion was stopped. This finding is consistent with a reduced pyruvate utilization which would be expected to result in an increased production of lactate. Phenylpyruvate, at the concentrations used in this study, did not affect the activity of phosphoenolpyruvate carboxykinase in agreement with the data of Seubert and Huth (1965). Since the immediate fate of pyruvate during gluconeogenesis is its carboxylation to oxalacetate as well as its oxidation, the data do not indicate whether the inhibition of one or both of these processes was responsible for the reduced glucose production. Two approaches were used to assess the extent to which these reactions were affected by phenylpyruvate and the relative importance of these interactions to overall glucose production in the perfused liver. First, the capacity of liver homogenates to carboxylate and decarboxylate pyruvate in the presence of various concentrations of substrate and inhibitor was tested. Secondly, gluconeogenesis from pyruvate by the perfused liver was measured under conditions known (Jagow *et al.*, 1968; Stucki *et al.*, 1972) to suppress pyruvate metabolism *via* pyruvate dehydrogenase.

Effect of Phenylalanine and Phenylpyruvate on Pyruvate Carboxylation by Liver Homogenates. At a pyruvate concentration of 5 mM which ensures maximum fixation of $H^{14}CO_3^-$ in the system used the carboxylation of pyruvate was progressively inhibited by increasing the concentration of phenylpyruvate from 0 to 5 mM (Figure 4). In this system, ^{14}C is incorporated into organic acids, principally citrate and malate (Walter *et al.*, 1966). A 50% inhibition of this process was attained at a phenylpyruvate concentration of 0.75 mM. When the concentration of pyruvate was lowered to 0.5 mM, the carboxylation process was inhibited by 50% at a phenyl-

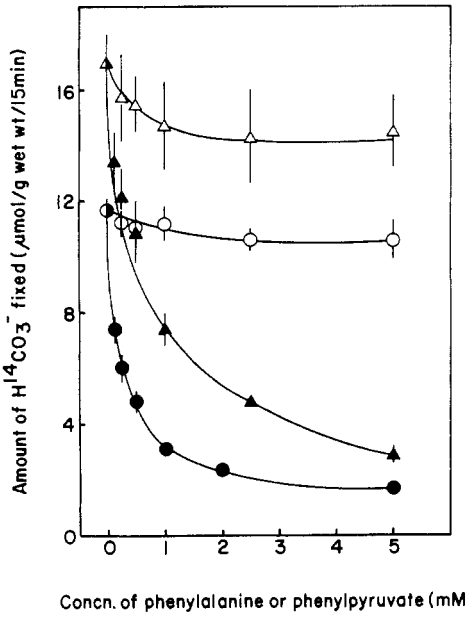

FIGURE 4: Effect of various concentrations of L-phenylalanine and phenylpyruvate on the fixation of $H^{14}CO_3^-$ in the presence of pyruvate by rat liver homogenates. The reaction mixture contained, in a final volume of 1 ml, 6.6 mM potassium phosphate buffer (pH 7.4), 6.6 mM triethanolamine buffer (pH 7.4), 4 mM ATP (potassium salt), 10 mM $MgCl_2$, 10 mM $KH^{14}CO_3$ (1 μCi), and 157 mM sucrose. Potassium pyruvate was added at either 0.5 mM (circles) or 5 mM (triangles). L-Phenylalanine (○,△) or phenylpyruvate (●,▲) was added to give the final concentrations indicated. The reaction was started by adding liver homogenates (approximately 10 mg of tissue) and the incubation was carried out for 15 min at 37°. Points plotted are the means ±SEM (vertical bars) of four experiments.

pyruvate concentration of 0.25 mM. In contrast, phenylalanine had no appreciable effect.

Effect of Phenylalanine and Phenylpyruvate on the Oxidation of [1-^{14}C]Pyruvate and [1-^{14}C]α-Ketoglutarate by Liver Homogenates. Pyruvate oxidation *via* pyruvate dehydrogenase was measured by following the decarboxylation of [1-^{14}C]-pyruvate to $^{14}CO_2$. The addition of 1 mM phenylpyruvate inhibited $^{14}CO_2$ production from pyruvate by 15% irrespective of the pyruvate concentration in the incubation medium (Table II). This inhibition increased to 40% when the concentration of phenylpyruvate was raised to 5 mM. The addition of phenylalanine up to 5 mM had no influence on the decarboxylation of pyruvate. Gluconeogenesis from a variety of substrates is supported by the activity of the tricarboxylic acid cycle. Since glucose production from propionate, which is not metabolized *via* pyruvate, was also inhibited by phenylpyruvate (Table I) we tested the effect of this inhibitor on the oxidation of [1-^{14}C]α-ketoglutarate. This reaction is analogous to that of pyruvate oxidation and, as can be seen in Table II, was markedly inhibited by phenylpyruvate but not by phenylalanine. At 0.5 and 5 mM [1-^{14}C]α-ketoglutarate, phenylpyruvate at concentrations of 1 and 5 mM reduced the oxidation of the substrate by about 40 and 70%, respectively. It should be noted that at similar substrate and inhibitor concentration, phenylpyruvate exerted greater inhibition of the oxidation of [1-^{14}C]α-ketoglutarate than on the oxidation of [1-^{14}C]pyruvate.

Effect of Phenylpyruvate on Glucose Production in the Presence of Octanoate or β-Hydroxybutyrate. Figure 5A shows that in the presence of 0.2 mM octanoate, phenylpyruvate (2 mM) inhibited gluconeogenesis from pyruvate by 25% as

TABLE II: Effect of Phenylalanine and Phenylpyruvate on the Decarboxylation of [1-^{14}C]Pyruvate and [1-^{14}C]α-Ketoglutarate to $^{14}CO_2$ by Rat Liver Homogenates.[a]

Substrate (mM)	Additions (mM)	Production of $^{14}CO_2$ from [1-^{14}C]Pyruvate or [1-^{14}C]α-Ketoglutarate: μmol/min per g of Tissue	% of Control
[1-^{14}C]Pyruvate (0.5)	None	2.29 ± 0.09	100
	L-Phenylalanine (1)	2.08 ± 0.18	91
	L-Phenylalanine (5)	2.27 ± 0.10	99
	Phenylpyruvate (1)	1.94 ± 0.16	85
	Phenylpyruvate (5)	1.41 ± 0.15	62
[1-^{14}C]Pyruvate (5.0)	None	2.17 ± 0.07	100
	L-Phenylalanine (1)	2.02 ± 0.11	93
	L-Phenylalanine (5)	1.97 ± 0.05	91
	Phenylpyruvate (1)	1.83 ± 0.15	84
	Phenylpyruvate (5)	1.31 ± 0.21	60
[1-^{14}C]Keto-glutarate (0.5)	None	2.62 ± 0.21	100
	L-Phenylalanine (1)	2.19 ± 0.14	84
	L-Phenylalanine (5)	2.08 ± 0.43	79
	Phenylpyruvate (1)	1.45 ± 0.45	55
	Phenylpyruvate (5)	0.64 ± 0.08	24
[1-^{14}C]α-Keto-glutarate (5.0)	None	2.98 ± 0.27	100
	L-Phenylalanine (1)	2.72 ± 0.32	91
	L-Phenylalanine (5)	2.86 ± 0.33	96
	Phenylpyruvate (1)	2.00 ± 0.16	67
	Phenylpyruvate (5)	1.01 ± 0.32	34

[a] The reaction mixture contained, in a final volume of 3 ml, 6.6 mM potassium phosphate buffer (pH 7.4), 6.6 mM Triethanolamine buffer (pH 7.4), 10 mM $MgCl_2$, 2 mM malate, 157 mM sucrose, and [1-^{14}C]pyruvate (specific radioactivity; 40 dpm/nmol) or [1-^{14}C]α-ketoglutarate (specific radioactivity 100 dpm/nmol) as indicated. L-Phenylalanine and phenylpyruvate were added to the incubation medium to give the final concentrations indicated. The reaction was started by adding liver homogenates (approximately 20 mg of tissue) and the incubation was carried out in a sealed flask for 15 min at 37°. Values given are the means ±SEM for four or five animals.

opposed to a 55% inhibition noted in the absence of the fatty acid. Since the oxidation of octanoate generates acetyl-CoA, an activator of pyruvate carboxylase, one may assume that the level of this effector was not limiting and therefore that the decrease in gluconeogenesis in the presence of octanoate represented in part glucose production under a condition where pyruvate oxidation was suppressed (Jagow *et al.*, 1968; Stucki *et al.*, 1972). However, the oxidation of octanoate also generates NADH and it is conceivable that this NADH resulted in the conversion of phenylpyruvate to phenyllactate and consequently relieved the inhibition. This seems unlikely because the conversion of phenylpyruvate to phenyllatate by lactate dehydrogenase occurs very slowly (Patel, 1972). Also when NADH was generated from β-hydroxybutyrate so as to avoid changes in acetyl-CoA levels, the increase in glucose production was comparable to that induced by 0.2 mM

TABLE III: Adenine Nucleotide Levels and Glucose Production in Perfused Livers Metabolizing Pyruvate in the Presence and Absence of Phenylpyruvate.[a]

Metabolite	Phenylpyruvate Absent	Phenylpyruvate Present	*P* Value
	μmol/g wet wt		
ATP	2.259 ± 0.110	1.941 ± 0.091	<0.05
ADP	0.711 ± 0.040	0.630 ± 0.042	n.s.
AMP	0.190 ± 0.017	0.210 ± 0.018	n.s.
	μmol/min per g wet wt		
Glucose	0.266 ± 0.010	0.149 ± 0.007	<0.001

[a] Livers from fasted rats were perfused with pyruvate (2 mM) after a 30-min pre-perfusion with Krebs–Ringer bicarbonate buffer. The livers were freeze clamped at the 65th min of perfusion. Phenylpyruvate was introduced into the perfusion system at the 45th min. ATP, ADP, and AMP were determined in the neutralized extracts of the frozen liver. Glucose was measured in the perfusate and the rates given represent glucose production at the time of the freeze clamps. Values given are the means ± SEM for eight livers. n. s. = not significant.

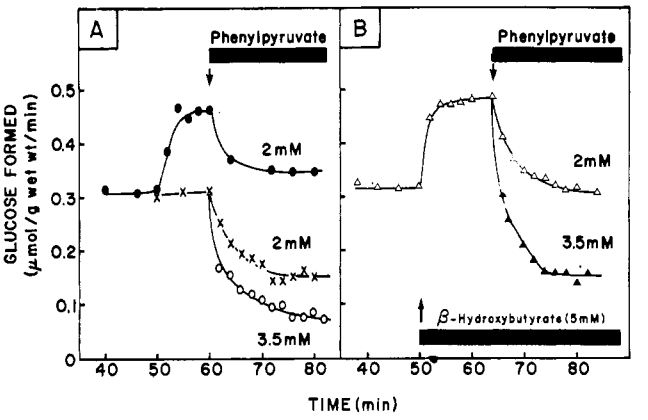

FIGURE 5: Influence of octanoate and β-hydroxybutyrate on the phenylpyruvate-induced inhibition of gluconeogenesis from pyruvate by the perfused rat liver. In part A, livers were perfused for 20 min with pyruvate (2 mM) followed for another 25 min with either 2 (X) or 3.5 mM (○) phenylpyruvate, or with pyruvate plus octanoate (0.2 mM) followed by 20 min of perfusion with 2 mM phenylpyruvate (●). In part B, β-hydroxybutyrate was introduced at 50 min and maintained at 5 mM by constant infusion throughout the experiment. Phenylpyruvate (2 Δ or 3.5 ▲ mM) was infused as indicated in the figure. Points plotted are the means of four to six experiments.

octanoate (Figure 5B) and in this case, the inhibition of gluconeogenesis by 2 mM phenylpyruvate was about 35% as compared to 25% in the presence of octanoate (Figure 5A). Secondly when saturating concentrations (3.5 mM or higher) of phenylpyruvate were used, the inhibition of glucose production was about the same (70–75%) with or without an added source of NADH.

An examination of the tissue metabolite levels in livers perfused with phenylpyruvate revealed no appreciable change in the concentrations of malate, phosphoenolpyruvate, 2-phosphoglycerate, and 3-phosphoglycerate. However, the decrease in glucose production was associated with a fall in the tissue concentrations of ATP (Table III). The levels of ADP and AMP remained unchanged.

Discussion

Krebs and de Gasquet (1964) first demonstrated the inhibition of gluconeogenesis by certain α-keto acids. In their studies with rat kidney cortex slices, glucose production from a variety of substrates was inhibited by phenylpyruvate but the mechanism of this inhibition was not investigated. Seubert and Huth (1965) also reported the inhibition by phenylpyruvate of gluconeogenesis from lactate and fumarate in pigeon liver homogenates and attributed it to inhibition of pyruvate carboxylase. Phenylpyruvate has also been shown to inhibit rat brain (Patel, 1972) and chicken liver (Scrutton *et al.*, 1969) pyruvate carboxylase. This mechanism appears inadequate to account for the inhibition of gluconeogenesis from malate, propionate, α-ketoglutarate, fumarate, and glutamate (Krebs and de Gasquet, 1964; see also Table I) since these substrates are not converted to glucose *via* pyruvate carboxylase.

In this paper we have examined additional sites of interaction of the inhibitor. Apart from the pyruvate carboxylase reaction, other sites of interaction are at the pyruvate and α-ketoglutarate dehydrogenase complexes. The data in Table II show that CO_2 production from [1-^{14}C]pyruvate was inhibited by 15 and 40% when the phenylpyruvate concentration in the medium was 1 and 5 mM, respectively. Inhibition of the pyruvate dehydrogenase complex would result in decreased production of acetyl-CoA, NADH, and ATP. Although a net generation of mitochondrial NADH is required during gluconeogenesis from pyruvate (Krebs *et al.*, 1967), decreased availability of NADH is unlikely to be an inhibitory event since glucose production from lactate (Krebs and de Gasquet, 1964) is also inhibited.

The importance of the inhibition of the pyruvate dehydrogenase reaction can be seen in the perfused liver synthesizing glucose from pyruvate in the presence of octanoate or β-hydroxybutyrate (Figure 5). The oxidation of fatty acid would generate not only NADH but also ATP and acetyl-CoA, thus sparing pyruvate oxidation and providing acetyl-CoA required to activate pyruvate carboxylase. In the presence of octanoate, the inhibition of gluconeogenesis by phenylpyruvate was reduced from 55 to 25% (Figure 5A). The data do not indicate the mechanism of inhibition by phenylpyruvate of pyruvate dehydrogenase complex but it appears that phenylpyruvate inhibits hepatic pyruvate carboxylase by competing with pyruvate (Figure 4; Seubert and Huth, 1965; Scrutton *et al.*, 1969). In the perfusion experiments shown in Figure 5A, this competition cannot be obviated by the inclusion of octanoate, and may account for the residual 25% inhibition of gluconeogenesis. However, the fact that glucose production from substrates which are not necessarily metabolized *via* these two enzymes was inhibited to varying degrees by phenylpyruvate (Table I) and other α-keto acids (Krebs and de Gasquet, 1964) suggests that perhaps these acids also interfere with metabolism at a site which is vital to gluconeogenesis from various substrates.

In this regard, the α-keto acids may interfere with the tricarboxylic acid cycle activity by competing with the α-keto acid substrates of the pyruvate and α-ketoglutarate dehydrogenases. The enzyme complexes of these two dehydrogenases are markedly similar (Sanadi *et al.*, 1952; Ishikawa *et al.*, 1966). It is apparent from Table II that phenylpyruvate is also a potent inhibitor of α-ketoglutarate decarboxylation. Inhibition at other enzymatic steps cannot, however, be ruled out. Land and Clark (1973) have recently shown that phenyl-

pyruvate also inhibits cerebral citrate synthase. The metabolism of branched-chain amino acids proceeds *via* initial conversion to α-keto acid derivatives. The data of Kanzaki *et al.* (1969) provide evidence of inhibition by α-ketoisocaproic acid of the pyruvate and α-ketoglutarate dehydrogenase complexes from pig heart. Other investigators have also reported the inhibition by α-ketoisocaproic acid of the oxidative decarboxylation of pyruvate and α-ketoglutarate in bovine, chicken, and rat liver and brain preparations (Bowden *et al.*, 1970, 1971; McArthur and Bowden, 1972; Johnson and Connelly, 1972). This inhibition is comparable to the effect of phenylpyruvate on the decarboxylation of pyruvate and α-ketoglutarate (Table II). Consequently, these α-keto acids may be expected to generally slow down tricarboxylic acid cycle activity and result in decreased availability of energy. This idea is supported by the data in Table III which shows reduced levels of ATP in livers perfused with phenylpyruvate. This mechanism may explain in part the inhibition of glucose production from malate, propionate, glutamate, and α-ketoglutarate since a common feature of glucose synthesis from these substrates is the dependence on the mitochondrial generation of energy equivalents. However, it cannot be deduced from the present study whether the reduced tissue levels of ATP reflects reduced generation of energy or whether it reflects the reduced biosynthetic activity of the organ. The branched-chain α-keto acids and phenylpyruvate were tested by Krebs and de Gasquet (1964) and found to inhibit to varying degrees gluconeogenesis from lactate and fumarate in rat kidney cortex slices. In their study, the most potent inhibitor was phenylpyruvate.

Phenylketonuria is characterized by marked elevation of plasma levels of phenylalanine of the order of 2–5 mM and the excretion of phenylpyruvic acid and its other metabolites in the urine. The intracellular concentration of phenylpyruvate is not known but in untreated phenylketonuric patients, its plasma concentration is approximately 0.1 mM (Jervis, 1952). This may be expected to vary depending on the rate of excretion *via* the kidney. In rat (Figure 4) as well as human liver (Sutnick *et al.*, 1972) homogenates, a similar concentration of phenylpyruvate inhibits pyruvate carboxylation, and in rat kidney cortex slices, 0.4 mM phenylpyruvate inhibits gluconeogenesis by about 70% even when the substrate (lactate) concentration is 10 mM (Krebs and de Gasquet, 1964). Hypoglycemia is not generally associated with phenylketonuria. However, if Weber's hypothesis (Weber *et al.*, 1970) that phenylalanine and phenylpyruvate inhibit glycolysis in brain and other tissues is correct, then a reduction of hepatic or renal gluconeogenesis by phenylpyruvate in phenylketonuric patients may be compensated by a decreased glucose utilization by extrahepatic tissues. Alternatively, it may be that the inhibition of gluconeogenesis, if it occurs *in vivo* with phenylpyruvate, is insufficient to compromise the capacity of the liver to maintain adequate blood glucose concentration.

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References

Arinze, I. J., Garber, A. J., and Hanson, R. W. (1973), *J. Biol. Chem. 248*, 2266.

Bickel, H., Hudson, F. P., and Woolf, L. I., Ed. (1971), Phenylketonuria, Stuttgart, Gerorge Thieme Verlag, p 1.

Bergmeyer, H. U., Ed. (1963), Methods in Enzymatic Analysis, New York, N. Y., Academic.

Bowden, J. A., Brestel, E. P., Cope, W. T., McArthur, C. L., III, Westfall, D. N., and Fried, E. M. (1970), *Biochem. Med. 4*, 69.

Bowden, J. A., McArthur, C. L., III, and Fried, M. (1971), *Biochem. Med. 5*, 101.

Hill, J. B., and Kessler, G. (1966), *J. Lab. Clin. Med. 57*, 970.

Hohorst, H. J. (1965), *in* Methods in Enzymatic Analysis, Bergmeyer, H. U., Ed., New York, N. Y., Academic.

Hopper, S., and Segal, H. L. (1962), *J. Biol. Chem. 237*, 3189.

Ishikawa, E., Oliver, R. M., and Reed, L. J. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 534.

Jagow, G. V., Westermann, B., and Wieland, O. (1968), *Eur. J. Biochem. 3*, 512.

Jervis, G. A. (1952), *Proc. Soc. Expt. Biol. Med. 81*, 715.

Jervis, G. A. (1953), *Proc. Soc. Expt. Biol. Med. 82*, 514.

Johnson, W. A., and Connelly, J. L. (1972), *Biochemistry 11*, 2416.

Jomain-Baum, M., Garber, A. J., Farber, E., and Hanson, R. W. (1973), *J. Biol. Chem. 248*, 1536.

Kanzaki, T., Hayakawa, T., Hamada, M., Fukoyoshi, Y., and Koike, M. (1969), *J. Biol. Chem. 244*, 1183.

Knox, W. E., and Pitt, B. M. (1957), *J. Biol. Chem. 225*, 675.

Krebs, H. A., and de Gasquet, P. (1964), *Biochem. J. 90*, 149.

Krebs, H. A., Gascoyne, T., and Notton, B. M. (1967), *Biochem. J. 102*, 275.

Land, J. M., and Clark, J. B. (1973), *Biochem. J. 134*, 545.

McArthur, C. L., III, and Bowden, J. A. (1972), *Int. J. Biochem. 3*, 193.

Meister, A. (1965), Biochemistry of the Amino Acids, Vol. II, New York, N. Y., Academic, p 899.

Meister, A., Udenfriend, S., and Bessman, S. P. (1956), *J. Clin. Invest. 35*, 619.

Miller, J. E., and Litwack, G. (1971), *J. Biol. Chem. 246*, 3234.

Patel, M. S. (1972), *Biochem. J. 128*, 677.

Patel, M. S., and Hanson, R. W. (1970), *J. Biol. Chem. 245*, 1302.

Ross, D. B., Hems, R., and Krebs, H. A. (1967), *Biochem. J. 102*, 942.

Sanadi, D. R., Littlefield, J. W., and Bock, R. M. (1952), *J. Biol. Chem. 197*, 851.

Schnitger, H., Scholz, R., Bücher, Th., and Lubbers, D. W. (1965), *Biochem. Z. 341*, 334.

Scholz, R. (1968), *in* Stoffwechsel der perfundierten Leber, Staib, W., and Scholz, R., Ed., Berlin, Springer-Verlag, p 25.

Scholz, R., Schwarz, F., and Bücher, Th. (1966), *Z. Klin. Chem. 4*, 179.

Scrutton, M. C., Olmsted, M. R., and Utter, M. F. (1969), *Methods Enzymol. 13*, 235.

Seubert, W., and Huth, W. (1965), *Biochem. Z. 343*, 176.

Shrawder, E., and Martinez-Carrion, M. (1972), *J. Biol. Chem. 247*, 2486.

Söling, H. D., Willms, B., Kleineke, H., and Gehlhoff, M. (1970), *Eur. J. Biochem. 16*, 280.

Stucki, J. W., Barwand, F., and Walter, P. (1972), *Eur. J. Biochem. 27*, 181.

Sutnick, M. R., Grover, W. D., and Patel, M. S. (1972), *Pediatric Res. 6*, 432, Abstr.

Utena, H., and Saito, T. (1951), *Psychiat.-Neurol. Japon. 53*, 365.

Walter, P., Peatkau, V., and Lardy, H. A. (1966), *J. Biol. Chem. 241*, 2523.
Weber, G., Glazer, R. I., and Ross, R. A. (1970), *Advan. Enzyme Regul. 8*, 13.
Wollenberger, A., Ristau, O., and Schoffa, G. (1960), *Pfluegers Arch. Gesamte Physiol. Menschen Tiere 270*, 399.

Polyamine Accumulation During Lymphocyte Transformation and Its Relation to the Synthesis, Processing, and Accumulation of Ribonucleic Acid†

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ABSTRACT: Large increases in the cellular levels of putrescine, spermidine, and spermine, as well as elevations of cellular RNA and protein, were found during concanavalin A-induced transformation of small lymphocytes isolated from bovine lymph nodes. The magnitude of these changes was: putrescine, 10–15-fold; spermidine, 5–7-fold; spermine, 2–2.5-fold; RNA, 3–5-fold; and protein, 2–3-fold. A careful analysis of the initial sequence of changes showed that the cellular RNA level was elevated within 6 hr of lymphocyte stimulation, whereas putrescine did not increase significantly until 8 hr, and elevations in the spermidine and spermine level were not observed until 10 hr.

Methylglyoxal bis(guanylhydrazone), a potent inhibitor of *S*-adenosyl-L-methionine decarboxylase, completely prevented intracellular accumulation of spermidine and spermine in cultures of transforming lymphocytes. No abnormalities in synthesis, processing, and accumulation of RNA were observed in these cultures. RNA accumulation was unchanged over a 40-hr interval and the absolute rate of RNA synthesis, estimated by measuring the incorporation of [8-^{3}H]adenosine into RNA and the specific activity of the ATP pool, doubled after 20 hr of concanavalin A treatment in the presence or absence of spermidine and spermine accumulation. All classes of RNA were synthesized (in the same proportion as in controls) in the polyamine-deficient cultures and the rate of processing of rRNA precursors was not altered. These results indicate that increases in the spermidine and/or spermine levels are not essential in mediating the large increases in cellular RNA observed during lymphocyte transformation.

The aliphatic polyamines spermidine and spermine, and their precursor putrescine, have been implicated in numerous growth processes (reviewed by Cohen, 1971). In the time since parallel increases in spermidine and RNA were first reported by Raina (1963) in developing chick embryo and subsequently by Dykstra and Herbst (1965) in regenerating rat liver, hypotheses casually relating polyamine levels and RNA metabolism have been frequently advocated. For example, it has been suggested that polyamines may serve a role in the regulation of RNA metabolism in eukaryotes (Caldarera *et al.*, 1965; Russell, 1970; Raina and Jänne, 1970; Russell and Lombardini, 1971), and it has recently been proposed that polyamines are specific regulators of rRNA synthesis (Russell and McVicker, 1973). In contrasting proposals, polyamines were suggested to increase in parallel with RNA in order to neutralize a fraction of the RNA phosphate residues and stabilize the RNA (Raina *et al.*, 1966; Raina and Jänne, 1968). Unfortunately, even in the most thoroughly studied system, regenerating liver, it is not yet clear whether spermidine accumulation precedes or occurs simultaneously with RNA accumulation (Dykstra and Herbst, 1965; Raina *et al.*, 1966; Raina *et al.*, 1970; Russell *et al.*, 1970; Russell and Lombardini, 1971). Obviously, a definite answer to this question is critical in formulating models for polyamine action, since polyamines should increase prior to RNA if they are serving a regulatory role.

Small lymphocytes, stimulated to transform by concanavalin A,[1] present an excellent system in which to define the relationship between polyamine and nucleic acid accumulation. Most small lymphocytes are normally quiescent both *in vivo* and in culture (Nowell, 1960; Robbins, 1964). However, upon stimulation *in vitro* by Con A or phytohemagglutinin (*Phaseolus vulgaris*), lymphocytes are transformed into large blast cells capable of division (Robbins, 1964; Ling, 1968; Powell and Leon, 1970; Novogradsky and Katchalski, 1971). Among the early events in lymphocyte transformation are large increases in the rate of uptake of radioactive precursors and their incorporation into RNA and protein (Kay and Korner, 1966; Kay, 1968; Lucas, 1967; Hausen *et al.*, 1969). This is followed by net RNA and protein accumulation at later times (Hausen *et al.*, 1969; Fisher and Mueller, 1969; Forsdyke, 1967). DNA synthesis begins approximately 24 hr after stimulation (Powell and Leon, 1970; Novogradsky and Katchalski, 1971; Loeb and Agarwal, 1971) and the cells begin dividing on the second and third days of culture (Bender and Prescott, 1962).

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[1] Abbreviations used are: ConA, concanavalin A; MGBG, methylglyoxal bis(guanylhydrazone).