Gluconeogenesis from Pyruvate in Isolated Perfused Rat Liver*

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ABSTRACT: Glucose biosynthesis from 2.0 mM pyruvate was studied in isolated perfused livers from fasted rats. In the presence of quinolinate, a specific inhibitor of phosphoenolpyruvate carboxykinase, the rate of glucose synthesis during the hour after the addition of pyruvate to the perfusate was 55% of that in control livers. With pyruvate-*2*-^{14}C as substrate, quinolinate-inhibited liver utilized pyruvate carbon for glucose synthesis to the extent of 60% of that by control liver. This could be increased to 80% when the quinolinate-treated liver was also perfused with caprylate at physiologic concentration. After 5 to 10 min of perfusion with pyruvate free of added fatty acids, large amounts of phosphoenolpyruvate, 2-phosphoglycerate, and 3-phosphoglycerate accumulated. An increase in these metabolites also occurred in the presence of quinolinate. That the phosphoenolpyruvate carboxykinase inhibition was substantial was supported by the demonstration that, when perfused for 30 min with labeled pyruvate or 60 min with labeled alanine, quinolinate-treated livers used these substrates selectively, producing less glucose but of greater apparent specific activity than that produced by control livers. These data suggest that, in the conversion of pyruvate into glucose under the conditions of our experiments, an alternative pathway which does not require phosphoenolpyruvate carboxykinase activity may be open to pyruvate.

Various hepatic glucogenic intermediates were isolated and their radioactivity was measured after perfusions with ^{14}C-labeled pyruvate for 3, 5, or 10 min. The specific radioactivities of pyruvate and oxaloacetate could not be measured and they were assumed to be equivalent to those of their reduction products, lactate and malate, respectively. In all cases the specific radioactivity of 3-phosphoglycerate exceeded that of malate but was comparable to that of lactate. If oxaloacetate is not compartmented, these data are consistent with the contention that synthesis of 3-phosphoglycerate from pyruvate does not proceed solely through the phosphoenolpyruvate carboxykinase reaction. In all cases there was a discrepancy between the specific radioactivities of 3-phosphoglycerate and of phosphoenolpyruvate of at least 2:1, and this was not rectified by the addition of caprylate. If phosphoenolpyruvate is indeed the precursor of 3-phosphoglycerate in the gluconeogenic pathway from pyruvate, as is widely held, then the data suggest that phosphoenolpyruvate might be compartmented. Alternatively, under the relatively unphysiologic conditions of our perfusions, the isolated liver can convert pyruvate into 3-phosphoglycerate by a pathway in which neither phosphoenolpyruvate nor phosphoenolpyruvate carboxykinase activity is obligatory. It was demonstrated that caprylate did activate the major pathway initiated by the enzyme pyruvate carboxylase, but not necessarily to the exclusion of any other pathway that might be operative.

The initial reaction in the biosynthesis of glucose from pyruvate by liver is widely held to be the formation of oxaloacetate, which is catalyzed by the enzyme, pyruvate carboxylase (Utter and Keech, 1960, 1963; Keech and Utter, 1963). When they discovered, in 1935, that bacteria can fix CO_2 onto pyruvate, Wood and Werkman predicted that such CO_2-fixing reactions would be found in animal tissues. This was followed by the demonstration that intact rats can incorporate administered bicarbonate-^{11}C into liver glycogen (Solomon *et al.*, 1941; Vennesland *et al.*, 1942). That bicarbonate can serve as a carbon source for glucose synthesis was confirmed by data from liver-slice experiments (Topper and Hastings, 1949; Hiatt *et al.*, 1958). Added support for the scheme as it is now accepted was obtained from the labeling patterns observed in glucose moieties of glycogen derived from pyruvate (Topper and Hastings, 1949) and lactate (Lorber *et al.*, 1950) specifically labeled with isotopic carbon. Randomization of the label in glucose has been used as evidence that oxaloacetate is an obligatory intermediate in gluconeogenesis from pyruvate and, indeed, randomization of the label between the two halves of the four-carbon dicarboxylic acids, oxaloacetate, malate, and aspartate, has been demonstrated to occur rapidly as originally postulated (Solomon *et al.*, 1941; Hoberman and D'Adamo, 1960d; Bloom and Foster, 1962; Haynes, 1965). The routes by which these four-carbon acids can be metabolized in the gluconeogenic process have been investigated (Kalckar, 1939; Utter and Kurahashi, 1954; Utter, 1963; Krebs, 1964; Haynes, 1965; Lardy *et al.*, 1965; Shrago and Lardy, 1966). Although the results of these investigations have not given direct and unequivocal proof that the four-carbon dicarboxylic acids are obligatory intermediates in the conversion of pyruvate into glucose, they have been in agreement with that widely accepted belief.

The formation of phosphoenolpyruvate directly from pyruvate, as by the reversal of the pyruvate kinase reaction, has been investigated in animal tissues. The data indicate that this is possible in muscle (Lardy and Ziegler, 1945;

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Hiatt *et al.*, 1958), in a reconstructed system (Krimsky, 1959), and in liver (Hoberman and D'Adamo, 1960a). We have sought to determine whether the dicarboxylic acids are essential and exclusive intermediates for carbohydrate synthesis from pyruvate by liver under all conditions or whether part of the pyruvate may be utilized by other pathways.

The use of quinolinate, which specifically inhibits phosphoenolpyruvate carboxykinase in the perfused rat liver (Veneziale *et al.*, 1967), and of extensive tracer methodology (Veneziale and Gabrielli, 1969)[1] has provided the opportunity to determine whether there is a route of gluconeogenesis from pyruvate which does not require phosphoenolpyruvate carboxykinase. This paper presents evidence that the isolated perfused rat liver can form glucose from pyruvate without prior formation of malate, aspartate, or phosphoenolpyruvate. The physiologic significance of this finding has yet to be determined.

Materials and Methods

Fasted (24 hr), male Sprague–Dawley rats were the source of erythrocytes and livers used in the perfusion experiments. The perfusate consisted of rat erythrocytes, from 75 ml of whole blood, suspended in Krebs–Ringer bicarbonate solution (Umbreit *et al.*, 1964) containing 3% (w/v) bovine serum albumin. The perfusion method, liver sampling techniques, and assays for glucose and various intermediates have been described (Veneziale *et al.*, 1967). The same criteria of viability of the liver were used. Perfusate flow was maintained at about 12 ml/min per 10 g of liver. In all cases the total volume of perfusate was 100 ml.

The perfusion experiments were conducted in pairs, one liver serving as a control. Technique was standardized and identical in all experiments. With zero time defined as the start of perfusion: (1) perfusate was sampled at 30, 60, 90, and 120 min for glucose determination. In experiments terminated at various times between 60 and 90 min, the final glucose determination was made on specimens taken at the termination time.

(2) When used, 0.24 mmole of quinolinic acid partially neutralized with an equivalent weight of NaOH was added to the perfusate at 30 min to give a concentration of 2.4 mM. Substrate (1.0 or 2.0 ml) was added at 60 min to give a final concentration of 2.0 mM.

(3) A 0.8- to 1.0-g specimen was taken from each liver after an appropriate interval of perfusion, instantly homogenized in dilute perchloric acid at $-5°$ in a tared tube, and weighed; a protein-free extract was made of it. These extracts were analyzed for the intermediates of gluconeogenesis, and those from isotopic experiments were also subjected to paper chromatography as described elsewhere (Veneziale and Gabrielli, 1969).

(4) In some experiments in which ^{14}C-labeled substrate was used, 4.0 ml of perfusate was taken at 120 min and the glucose was converted into glucose phenylosazone (Feller *et al.*, 1950) for the purpose of determining the ^{14}C incorporated into the total circulating glucose at that time. The 4.0 ml of perfusate was first deproteinized by the method of Somogyi (1945) using 10.0 ml of $Ba(OH)_2$ and $ZnSO_4$ solutions. The purified osazone (three recrystallizations) was dried and dissolved in 100% ethanol (approximately 100 μg/ml); 1 ml of this solution was added to 18.0 ml of toluene-based scintillation mixture (4.0 g of 2,5-diphenyloxazole, 100 mg of dimethyl 1,4-bis[2-(5-phenyloxazolyl)]benzene, and 50 g of naphthalene made to 1.0 l. with toluene) and counted in a Packard 3310 Tri-Carb scintillation counter with an efficiency of 80%. The latter, in this and in the case of all other scintillation mixtures, was determined from standard curves based on counts of external standards.

^{14}C-labeled substrate solutions were counted in the Tri-Carb also, using Bray's solution (Bray, 1960) except that dimethyl 1,4-bis[2-(5-phenyloxazolyl)]benzene was substituted for 1,4-bis[2-(5-phenyloxazolyl)]benzene.

Two different two-dimensional paper chromatographic procedures (Veneziale and Gabrielli, 1969) were devised expressly for this and other studies in the biochemistry of gluconeogenesis and provide good separation of the key compounds of gluconeogenesis. The individual species were detected by radioautography, and areas of paper to which they had localized were then excised and counted with an efficiency of 65% based on the counting of a standard serine-^{14}C solution on paper in the toluene-based scintillation mixture. The recoveries of the radioactivity of ^{14}C-labeled glutamate, malate, aspartate, 3-phosphoglycerate, and glucose separated by the methods used in this paper have been shown to be 85–90%; recovery of labeled phosphoenolpyruvate is at least 75%.

That our identification of 3-phosphoglycerate (2-phosphoglycerate) by systems 1 and 2 was correct was established in some experiments by the demonstration that, after rechromatography of an aliquot of extract appropriately treated with the terminal four glycolytic enzymes and the necessary cofactors and ions, the spot that allegedly was 3-phosphoglycerate (2-phosphoglycerate) on each of the radioautographs of untreated extracts then disappeared. System 2 was especially useful for this demonstration because the migration is not affected by the salts and proteins which are present. Concomitantly, a large portion of the radioactivity that disappeared from the 3-phosphoglycerate spot appeared at the site to which lactate localized on system 1 chromatograms.

The correct identification of 3-phosphoglycerate and other phosphate esters by chromatography system 1 was further established by combining the use of solvent A as described (Veneziale and Gabrielli, 1969) and high-voltage electrophoresis (Gilson Electrophorator, Model D) in a pyridine–acetate buffer (pH 3.0; 2.0 M in acetate) at 4500 V for 90 min. After development of a 16 by 22.5 in. paper in solvent A, sufficient paper was sewn to each narrow edge so that the chromatogram could be placed into the standard symmetric paper rack of the Electrophorator. The origin was placed in the cathode compartment.

For degradation studies, perfusate glucose was isolated in pure form in the following way. At 120 min the whole perfusate was deproteinated at 0° by the slow addition of 4.8 ml of 60% (w/v) perchloric acid with constant stirring.

[1] In that publication it was incorrectly stated that fructose diphosphate and 2-phosphoglycerate have the same mobilities by systems 1 and 2. Fructose diphosphate has the same R_F as 2,3-diphosphoglycerate, and 2-phosphoglycerate has the same R_F as 3-phosphoglycerate in the solvent systems described.

TABLE I: Rates of Gluconeogenesis and Metabolite Concentrations in Isolated Livers Perfused with Pyruvate or Alanine without and with Quinolinate.[a]

Quinolinate	0	+	0	+	0	+
Substrate	0	0	Pyr	Pyr	Ala	Ala
Perfusions (no.)	5	5	5	5	4	4
Ns Glc	77	36	92	51	92	30
	±16	±18	±16	±18	±26	±19
Lactate	0.80	0.42	0.54	0.73	0.86	0.65
	±0.39	±0.26	±0.16	±0.29	±0.42	±0.19
Pyruvate	0.073	0.11	0.088	0.22	0.14	0.22
	±0.042	±0.07	±0.029	±0.13	±0.07	±0.10
Aspartate	0.36	0.89	0.31	1.13	0.36	1.31
	±0.06	±0.17	+0.11	±0.35	±0.08	±0.06
Malate	0.15	0.38	0.15	0.41	0.18	0.41
	±0.02	±0.10	±0.05	±0.11	±0.03	±0.08
Phosphoenolpyruvate	0.13	0.058	0.13	0.094	0.17	0.053
	±0.03	±0.009	±0.06	±0.026	±0.03	±0.007
2-Phosphoglycerate	0.037	0.020	0.036	0.031	0.039	0.016
	±0.007	±0.004	±0.008	±0.008	±0.003	±0.003
3-Phosphoglycerate	0.35	0.15	0.35	0.22	0.40	0.14
	±0.07	±0.01	±0.12	±0.06	±0.04	±0.02

[a] Quinolinate was added at 30 min. Substrates were added at 60 min to a perfusate concentration of 2.0 mM. Data are shown as means ± std dev. Rates of gluconeogenesis are for the interval 60–120 min. Analyses for metabolites were done on extracts of liver specimens taken at 120 min. Rate of gluconeogenesis (Ns Glc) is in μmoles/10 g per 60 min. Metabolite concentrations are μmoles/g (wet wt). Pyr = pyruvate; Ala = alanine; Ns Glc = newly synthesized glucose.

The precipitate, collected by centrifugation, was washed with 50 ml and then with 20 ml of 6% perchloric acid. The supernatant fractions were combined and, with stirring at 0°, were slowly brought to pH 1.0 or less with 6.5 ml of 5.0 M K_2CO_3. After centrifugation and removal of the potassium perchlorate, the protein-free filtrate (approximately 130 ml) was subjected to a 24-hr continuous liquid–liquid extraction with diethyl ether in a Kutscher–Steudel extractor. Subsequently, the pH was adjusted to 2.2 with NaOH and the filtrate was passed through a column (22 × 1.5 cm) of Dowex AG 50W-X8 cation-exchange resin in hydrogen form. The desired fraction of eluate, about 130 ml, was identified by use of Tes-Tape (Eli Lilly Co.), lyophilized, and then brought to a volume of 10.0 ml at pH 5.5. This solution was passed through a column (40 × 2.5 cm) of Amberlite MB-3 prepared by carbonation of an aqueous suspension of the resin in a large beaker; the glucose could be collected salt free in a volume as little as 100 ml (if the upper portion of the resin was stirred to facilitate the escape of CO_2, a greater volume of eluate had to be collected to recover the glucose). The eluate was evaporated to about 0.5 ml in a rotary flash evaporator, taken up quantitatively in 6.0 to 7.0 ml, and analyzed for glucose. The average recovery of perfusate glucose in four samples was 58%.

The solution was treated as described by Blair and Segal (1960) to oxidize the glucose to potassium gluconate by the method of Moore and Link (1940). The gluconate was recrystallized once, collected in a sintered glass filter, and dried. Prepared in this way, each sample was found to be pure by two criteria: (1) decomposition occurred sharply at 175°, and (2) chromatography of each gluconate sample by systems 1 and 2 disclosed only one spot on each radioautograph. The specific activity of the gluconate was determined directly by counting known amounts in Bray's scintillation liquid.

A minimum of 20 mg of potassium gluconate was degraded by the procedure of Eisenberg (1954), except that the CO_2-trapping mixture consisted of 4.5 ml of methyl Cellosolve–ethanolamine (2:1) (Jeffay and Alvarez, 1961) in a Vigreaux-type CO_2 absorption tower. Carbon 1 was trapped during the periodate oxidation. Carbon 6 was isolated as formaldehyde by distillation from the periodate oxidation flask; this was oxidized to formate by I_2 and then to CO_2 by $HgCl_2$ which could be trapped as was carbon 1. The periodate oxidation, the oxidation of formaldehyde to formate, and the oxidation of formate to CO_2 each was permitted to proceed for 1 hr (Hankes *et al.*, 1969). The CO_2 absorption has been shown to be quantitative (Jeffay and Alvarez, 1961; L. Anderson, unpublished data). The trapping mixture was brought to 10.0 ml quantitatively with methyl Cellosolve and 1.0 ml of this was added to a scintillation vial containing 18.0 ml of a scintillation liquid modified slightly from the one described by Jeffay and Alvarez (1961) (toluene–methyl Cellosolve, 10.0:6.5). Counting efficiency in the Packard Tri-Carb exceeded 75%.

The solution remaining after the formaldehyde distillation contained carbons 2, 3, 4, and 5 as sodium formate. This solution was adjusted to pH 2.0 to 2.5 and transferred to a 50.0-ml volumetric flask with at least three washings of the undissolved salt remaining in the flask. After the solution was made up to 50.0 ml, 50- or 100-μl aliquots were counted in vials containing 18.0 ml of Bray's scintillation liquid to

TABLE II: Incorporation of ^{14}C of Alanine or Pyruvate into Glucose and Glucogenic Metabolites by Isolated Livers without and with Quinolinate.[a]

Experiment	299	300	293	294	309
Quinolate	0	+	0	+	+
Substrate					
Type	Ala-*U*-^{14}C	Ala-*U*-^{14}C	Pyr-*2*-^{14}C	Pyr-*2*-^{14}C	Pyr-*2*-^{14}C + caprylate
Sp act. $\times 10^{-5}$	2.2	2.2	2.1	2.1	2.1
Ns Glc (μmoles/10 g per 60 min)	77	6	86	77	85
^{14}C in Glc (dpm $\times 10^{-6}$/10 g) at 120 min[b]	8.2	2.8	16.7	10.1	13.4
Spec act. (dpm/μmole) at 60 min					
Ns Glc[c]	110,000	440,000	190,000	120,000	160,000
Lactate[d]	13,000	11,000	27,000	23,000	*e*
Malate	*e*	18,000	*e*	21,000	18,000
Aspartate	17,000	22,000	38,000	28,000	7,700
Glutamate	24,000	18,000	33,000	27,000	16,000

[a] Quinolinate was added at 30 min. Substrates were added at 60 min to give a perfusate concentration of 2.0 mM. Caprylate was added in 60-μmole quantities at 60, 75, 90, and 105 min. Rates of gluconeogenesis are for the interval 60–120 min. See Materials and Methods for details. For specific activity of lactate, malate, and glutamate, extract was subjected to paper chromatography system 1; for aspartate, extract was subjected to chromatography system 2. Concentrations of these compounds in extracts were determined enzymatically. Ala = alanine; Pyr = pyruvate; Ns Glc = newly synthesized glucose. [b] Counted as D-glucose phenylosazone. [c] Calculated by dividing total disintegrations per minute due to glucose at 120 min by micromoles of glucose formed during the interval 60–120 min. [d] Based on concentration of lactate. This ignores contamination by succinate which has the same mobility as lactate in system 1. This system was chosen because it produces a more confined spot of lactate than does system 2 and because sucrose can be ignored compared to the amount of lactate. [e] Not detectable.

establish the amount of radioactivity found in carbons 2, 3, 4, and 5 combined.

Results

Liver Glycogen. In specimens from eight livers excised at 25–33 min without additions to the perfusates, the mean (±std dev) glycogen content, measured as micromoles of glucose, was 12 ± 1/10 g of liver.

Effects of Quinolinate. GLUCOSE SYNTHESIS AND HEPATIC METABOLITES. The results after addition of pyruvate or alanine to the perfusates are shown in Table I. In the case of alanine, the presence of quinolinate decreased the rate of gluconeogenesis to a third of the control value; this rate was approximately the same as in the presence of quinolinate without added substrate. In the case of pyruvate, the inhibitor was not as effective. The effect of quinolinate on the pattern of metabolites was pronounced and as has been reported (Veneziale *et al.*, 1967). It also has been shown (Veneziale *et al.*, 1967) that, in the isolated liver perfused for 15 min with quinolinate, the assayable activity of phosphoenolpyruvate carboxykinase is altered. In experiments (not shown here) to see how quickly quinolinate altered the pattern of glucogenic metabolites as a measure of its onset of inhibition of phosphoenolpyruvate carboxykinase, 30 min after quinolinate was added to the perfusate there was an increase in the combined concentration of aspartate and malate even though substrate had not been added. The accumulation of these dicarboxylic acids is offered as evidence that phosphoenolpyruvate carboxykinase was substantially inhibited in the perfused livers by 60 min. This fact taken together with the gluconeogenesis data of Table I suggested that either pyruvate lessened the effectiveness of quinolinate or part of the pyruvate was converted into glucose by a pathway not involving phosphoenolpyruvate carboxykinase.

Considerable glucose synthesis occurred in the absence of added substrate, demonstrating that endogenous glucogenic precursors are readily available and utilized. Very likely an important precursor of glucose, at least in the experiments with quinolinate without added substrate, is serine. The conversion of this amino acid into glucose is not influenced by quinolinate (Veneziale *et al.*, 1969).

INCORPORATION OF ^{14}C OF ALANINE OR PYRUVATE INTO GLUCOSE. This was investigated to evaluate the essentiality of the four-carbon dicarboxylic acids in gluconeogenesis from these substrates. When the rate of conversion of oxaloacetate into phosphoenolpyruvate was decreased by quinolinate, there was a decrease in the amount of ^{14}C from alanine or pyruvate incorporated into glucose (Table II). However, when pyruvate-^{14}C was the substrate, the decrease was not as great as with alanine-^{14}C. These data again indicate that either pyruvate lessens the effectiveness of quinolinate or that phosphoenolpyruvate carboxykinase activity may not be essential for gluconeogenesis from pyruvate.

Specific Radioactivities of Newly Synthesized Glucose and Precursors. The results after 60 min of perfusion with alanine-

TABLE III: Concentrations of Metabolites as Function of Time after Addition of Pyruvate.[a]

Expt	Time (min)	Metabolites (μmoles/g of liver, wet wt)						
		Lactate	Pyr	Asp	Malate	Phosphoenol-pyruvate	2-Phospho-glycerate	3-Phospho-glycerate
				Quinolate, 0; Pyruvate, 2.0 mM				
376	61	0.18	0.13	0.25	0.089	0.20	0.052	0.39
375	62	0.56	0.19	0.37	0.081	0.27	0.062	0.52
376	65	0.99	0.76	0.33	0.081	0.46	0.11	0.66
375	68	0.51	0.35	0.25	0.081	0.37	0.076	0.79
347	70	0.79	0.22	0.33	0.16	0.46	0.11	0.99
347	90	0.43	0.085	0.33	0.13	0.17	0.044	0.41
				Quinolate, 0; Pyruvate, 0				
378	61	0.17	0.036	0.52	0.073	0.22	0.050	0.41
377	62	...	0.026	0.43	0.081	0.23	0.052	0.44
378	65	0.18	0.040	0.52	0.098	0.20	0.050	0.41
377	68	0.05	0.040	0.43	0.089	0.20	0.052	0.44
321	75	0.10	0.022	0.39	0.098	0.091	0.030	0.25
				Quinolate, 2.4 mM; Pyruvate, 2.0 mM				
84A	61	0.41	0.24	0.63	0.42	0.085	0.018	0.16
83A	62	0.36	0.25	0.60	0.32	0.12	0.024	0.20
84B	65	0.61	0.32	0.61	0.38	0.25	0.054	0.46
83B	68	0.64	0.20	0.60	0.27	0.18	0.040	0.35
353	70	1.29	0.55	0.66	0.21	0.19	0.080	0.54
320[b]	60	0.33	0.054	0.62	0.36	0.083	0.028	0.18
322[b]	75	0.43	0.038	0.74	0.18	0.060	0.014	0.17

[a] Quinolinate and pyruvate were added at 30 and 60 min, respectively. At times indicated, tissue specimens were taken and extracted with perchloric acid as described under Materials and Methods. Metabolites were assayed enzymatically. [b] Pyruvate = 0 in these experiments.

^{14}C or pyruvate-^{14}C are given in Table II. The specific activity of newly synthesized glucose at 120 min was large in each experiment relative to the specific activity of the precursors isolated in pure form (lactate, malate, aspartate, and glutamate). Our interpretation is that gluconeogenesis from these labeled precursors occurred to a much greater extent earlier in the perfusions than at 120 min.

High radioactivity was found on the paper chromatograms at a site corresponding to 3-phosphoglycerate and to phosphoserine as well as to a third, unidentified species. The three are inseparable by systems 1 and 2. However, by chromatoelectrophoresis these highly radioactive sites noted on chromatograms from the experiments of Table II have been found to be due primarily to the unknown compound but also to 3-phosphoglycerate. The unknown compound is not any of the following: phosphoserine, phosphohydroxypyruvate, 2-phosphoglycerate, 2,3-diphosphoglycerate, phosphoenolpyruvate, fructose diphosphate, fructose 6-phosphate, glucose 6-phosphate, phosphoethanolamine, or glycerophosphate.

Hepatic Metabolites at Short Intervals after Addition of Pyruvate. To gain insight into the gluconeogenic process immediately following the addition of 2.0 mM pyruvate to the perfusate, glucogenic metabolites were determined at frequent intervals shortly after the addition of substrate (Table III). The concentrations of phosphoenolpyruvate, 2-phosphoglycerate, and 3-phosphoglycerate increased within a few minutes of the start of perfusion with pyruvate. Phosphoenolpyruvate reached its maximal concentration at 65 min, corresponding to a time of maximal pyruvate accumulation. At 70 min, the syntheses of phosphoenolpyruvate, 2-phosphoglycerate, and 3-phosphoglycerate were still occurring actively although it is possible that their increased concentrations could have been due at least in part to the accumulation of 3-phosphoglycerate secondary to a DPNH:DPN ratio unfavorable to the reduction of that metabolite. It is notable that neither aspartate nor malate concentration increased during the pyruvate perfusions, even when phosphoenolpyruvate, 2-phosphoglycerate, and 3-phosphoglycerate accumulated. In the third section of Table III the data from expt 83 and 84 show that the isolated livers retained or formed phosphoenolpyruvate and 3-phosphoglycerate even when phosphoenolpyruvate carboxykinase was inhibited by quinolinate.

Perfusion with Pyruvate-3-^{14}C. INCORPORATION OF ^{14}C INTO GLUCOSE AFTER 30 MIN WITHOUT AND WITH QUINOLINATE. Experiments 347 and 348 (Table IV) are offered as a limited assessment of the extent of the phosphoenolpyruvate carboxykinase block caused by quinolinate. On the basis of specific activity of newly synthesized glucose at 90 min, quinolinate

caused a selective utilization of pyruvate-*3*-^{14}C for the glucose that was formed. Such a selectivity would be unlikely if pyruvate carbon flowed exclusively through a partially inhibited phosphoenolpyruvate carboxykinase reaction in its conversion into glucose. This selective utilization could be explained by the existence of an alternative route of glucose biosynthesis not requiring phosphoenolpyruvate carboxykinase activity. An alternative explanation is that shortly after addition of labeled pyruvate in expt 348, when labeled substrate was abundant, highly radioactive phosphoenolpyruvate was formed by means of a "leak" through partially inhibited phosphoenolpyruvate carboxykinase.

At 70 min specimens of liver were taken in expt 347 and 348 before ligatures were carefully placed at the cut ends. The remaining liver tissue, which in each case consisted of approximately 90% of the intact organ, remained normal by the criteria of viability previously stated (Veneziale *et al.*, 1967). The liver specimens were extracted with perchloric acid and the extracts were submitted to the paper chromatographic and electrophoretic methods described in Materials and Methods. The specific activity of phosphoenolpyruvate of liver was 53,000 dpm/μmole in expt 347 and 36,000 dpm/μmole in expt 348. These data fail to support the alternative explanation—a "leak"—for the results of expt 348.

The specific activity of other metabolites in the liver at 70 min in expt 347 were (dpm/μmole): lactate, 110,000; aspartate, 37,000; malate, 37,000; 2-phosphoglycerate, 75,000; 3-phosphoglycerate, 91,000. The discrepancy between the specific activities of the four-carbon dicarboxylic acids and of 3-phosphoglycerate can be taken as evidence that the former could not have been the major sources of phosphoglycerate carbon. The data appear to be inconsistent with popular concepts of gluconeogenesis from pyruvate. Taken at face value and without postulating compartmentation of oxaloacetate and phosphoenolpyruvate, the data suggest that 3-phosphoglycerate was formed by a pathway not requiring phosphoenolpyruvate activity.

TABLE IV: Effect of Quinolinate on Incorporation of ^{14}C from Pyruvate-*3*-^{14}C into Glucose.[a]

	Quinolinate Absent	Quinolinate Present
Experiment	347	348
^{14}C in perfusate Glc at 90 min[b] (dpm $\times 10^{-6}$)	16.0	7.3
Glc formed between 60 and 90 min[c] (μmoles)	61	17
Apparent sp act. of ns Glc (ns Glc dpm/μmole)	260,000	430,000

[a] Quinolinate and pyruvate (2.0 mM, 22.1 μCi) were added at 30 and 60 min, respectively. To determine specific activity of newly synthesized glucose (ns Glc), livers were sampled at 90 min and extracted with perchloric acid as described. Extracts were submitted to paper chromatographic procedures designed to separate glucose from all known metabolites of gluconeogenesis. After identification by radioautography, radioactivity in glucose spots was measured. From this and the glucose concentration in extract, specific activity was calculated. [b] Calculated by multiplying specific activity of glucose (Glc) by total circulating perfusate glucose. When this method was compared with osazone method for determining disintegrations per minute in perfusate glucose in seven different experiments after 60 min of perfusion with labeled serine, alanine, or pyruvate, mean of percentage difference (±std dev) was 7.1 ± 3.9. [c] Uncorrected for differences in liver weight. At 60 min liver weights were: expt 347, 11.50 g; expt 348, 8.84 g.

INCORPORATION OF ^{14}C INTO GLUCOGENIC METABOLITES AND GLUCOSE AFTER 3 AND 5 MIN. Livers were perfused with pyruvate of specific activity, 1.1×10^6 dpm/μmole, in an attempt to evaluate at earlier times the alleged alternative pathway(s) suggested by the data of Table IV. Extracts from expt 381, 379, 380, and 382 (Table V) were submitted to chromatoelectrophoresis to determine the specific activity of 3-phosphoglycerate. Figure 1 shows the radiochromatoelectropherogram of expt 381. Species **1**, **2**, **3**, **5**, and **6**, which are thought to have the same mobility by chromatography, migrated different distances toward the anode during electrophoresis. By comparison to standards, species **5** appears to be labeled 3-phosphoglycerate and species **6**, 2-phosphoglycerate. After counting, species **5** and **6** were examined by enzymic analysis of aqueous eluates from the paper; **5** was identified as 3-phosphoglycerate, but no detectable 3-phosphoglycerate was found in **6**.

A striking feature of the results was that, after only 3 min of perfusion with pyruvate-*3*-^{14}C, 23 compounds were found (Figure 1) to be labeled. Between 63 min and 65 min, the counts in total circulating glucose doubled and the effect of 1.8 mM caprylate was to increase gluconeogenesis sixfold above the control rate. The specific activity of malate and of phosphoenolpyruvate was approximately the same at 63 min but that of phosphoenolpyruvate was greater than that of malate at 65 min. At both 63 and 65 min the specific activity of 2-phosphoglycerate and of 3-phosphoglycerate was greater than that of malate.

At present we know of no direct evidence that malate or phosphoenolpyruvate is compartmented into two pools and, therefore, these data suggest the tentative hypothesis that, under these experimental conditions, the isolated liver can convert pyruvate (lactate) into 3-phosphoglycerate by a pathway which does not require phosphoenolpyruvate carboxykinase activity. Perhaps oxaloacetate as a key intermediate in the conversion of pyruvate into glucose might be channeled to a three-carbon glucogenic fragment other than phosphoenolpyruvate. In any case it is evident that the data of expt 381 and 379 do not conform to widely held concepts of the paths of pyruvate carbon in gluconeogenesis. More specifically, in the absence of added free fatty acid, the four-carbon dicarboxylic acids malate and aspartate played much less of a role than expected of them.

Glucagon was without a marked stimulatory effect on the rate of conversion of pyruvate into glucose. It will be the subject of separate reports. The effects of caprylate, which were conspicuous, can be explained in large part by its stimulation of pyruvate carboxylase activity and of glucose synthesis from 3-phosphoglycerate. As a result of the presence of caprylate, malate synthesis from pyruvate was augmented greatly, to the extent that it could have served as a major

TABLE V: Incorporation of ^{14}C into Glucogenic Metabolites and Glucose after Short Perfusion with Pyruvate-*3*-^{14}C.[a]

Experiment	381	379	380	382
Other addition	0	0	glucagan, 5 μg	caprylate, 0.18 mmole
Time (min)	63	65	65	65
Ala[b]				
dpm/μmole	590,000	690,000	680,000	500,000
μmoles/g	1.57	1.18	1.71	0.17
Lactate				
dpm/μmole	320,000	310,000	400,000	580,000
μmoles/g	0.78	0.68	0.64	0.58
Asp				
dpm/μmole	21,000	58,000	53,000	180,000
μmoles/g	0.39	0.41	0.35	0.45
Malate				
dpm/μmole	69,000	77,000	100,000	630,000
μmoles/g	0.071	0.098	0.071	0.49
Glutamate				
dpm/μmole	110,000	190,000	180,000	410,000
μmoles/g	0.96	0.98	0.70	1.97
Phosphoenolpyruvate				
dpm/μmole	71,000	120,000	120,000	320,000
μmoles/g	0.49	0.46	0.69	0.18
2-Phosphoglycerate[c]				
dpm/μmole	140,000	260,000	190,000	520,000
μmoles/g	0.092	0.088	0.12	0.036
3-Phosphoglycerate[c]				
dpm/μmole	140,000	320,000	250,000	640,000
μmoles/g	0.96	0.89	1.32	0.37
Total Glc (dpm × 10^{-6})[d]	6.7	14	16	83
CIT (dpm × 10^{-3}/g)[e]	16	33	38	400
α-KG (dpm × 10^{-3}/g)[e]	18	44	22	110
SUC (dpm × 10^{-3}/g)[e]	15	18	27	200
FUM (dpm × 10^{-3}/g)[e]	10	32	13	27

[a] Substrate was added to perfusate at 60 min (pyruvate-*3*-^{14}C, 2.0 mM, 99.6 μCi, specific activity = 1.1 × 10^6 dpm/μmole). Glucagon and capyrlate were also added at 60 min: time refers to time of liver sampling. Liver specimen was extracted with perchloric acid. See Materials and Methods for assay and chromatography procedures used for determining specific activity. [b] Assayed by means of the amino acid analyzer; others assayed enzymatically. [c] The two species were separated by chromatoelectrophoresis. [d] Calculated by multiplying specific activity of glucose by the total circulating perfusate glucose. Specific activity of glucose was determined by means of chromatography in system 2 as described in Table IV. Perfusate glucose was measured at 1 min after time of liver sampling. [e] Concentration not determined. CIT, citrate; α-KG, α-ketoglutarate; SUC, succinate; FUM, fumarate.

source of 3-phosphoglycerate carbon. In effect, caprylate activated the well-known pathway of glucose synthesis from pyruvate, which is initiated by the enzyme pyruvate carboxylase and which requires malate dehydrogenase and phosphoenolpyruvate carboxykinase activities. Why the specific activity of 3-phosphoglycerate was higher than that of lactate, as well as that of phosphoenolpyruvate, is not clear. Perhaps caprylate activated the alleged hypothetical route of 3-phosphoglycerate synthesis from pyruvate in addition to the well-known pathway. The relatively low specific activity of phosphoenolpyruvate cannot be ascribed to the poor recovery of that compound in chromatography.

The aspartate and malate of individual livers (Table V) were not labeled equally. The discrepancy could be related to the relatively large pool size of aspartate and to the relative rates at which these compounds are formed from endogenous carbon sources. The data indicate that, in these experiments, aspartate was not as important a glucose precursor as was malate.

Distribution of ^{14}C *in Glucose Synthesized from Pyruvate-2-*^{14}C. The data (Table VI) demonstrate randomization at some step(s) in the metabolism of pyruvate by livers treated with quinolinate, as has been demonstrated by others (Topper and Hastings, 1949) in the absence of quinolinate. Without randomization the glucose would contain no isotope in positions 1 and 6. Since we have postulated that, under

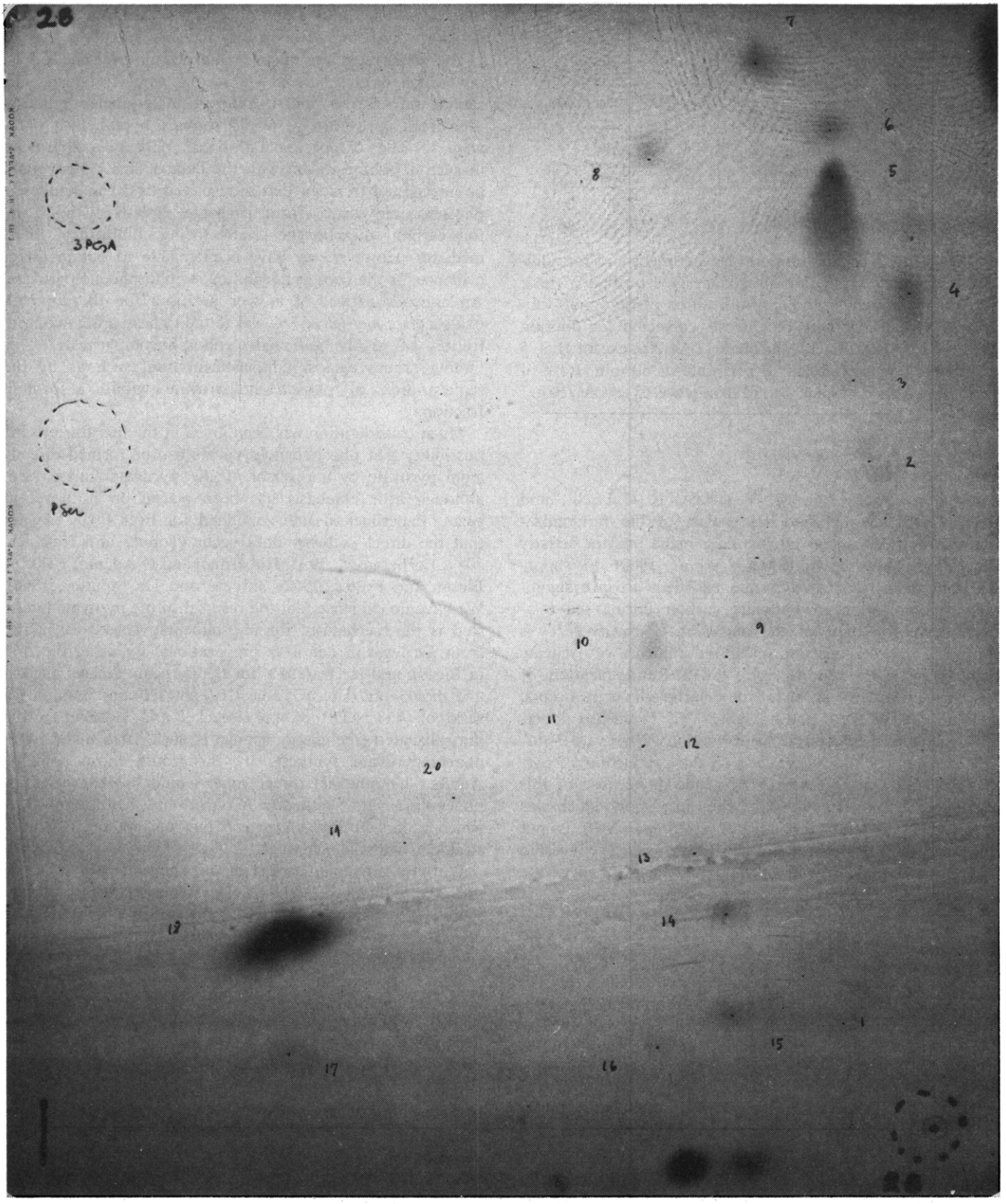

FIGURE 1: Radiochromatoelectropherogram of expt 381. See Materials and Methods for technique and Table V for description of experiment.

the conditions of our perfusions, the livers were capable of converting pyruvate into glucose by a pathway not requiring phosphoenolpyruvate carboxykinase activity, we must explain the observed distribution. At present this is poorly understood, especially in view of our observations that in gluconeogenesis from 2.0 mM L-serine-3-^{14}C, a process which does not appear to require phosphoenolpyruvate carboxykinase activity (Veneziale *et al.*, 1969), the isotope becomes ran-

TABLE VI: Distribution of ^{14}C in Glucose Synthesized from Pyruvate-*2*-^{14}C.[a]

Expt	Sp Act. of Glc as Osazone[b]	Potassium Gluconate (dpm/μmole)	Radioactivity (%) C-1	C-2, -3, -4, and -5	C-6
309	12,000	13,000	17.8	57.4	20.3
310[c]	3,400	4,100	21.1	50.3	26.6

[a] See Table II for description of experiment and for additional results. Degradation procedure is described in text. No attempt was made to confirm position of isotope in substrates. [b] Specific activity of glucose, corrected for dilution factor for preparation of gluconate. [c] As 309 except that 5 μg of glucagon was added to perfusate at 60 min. Rate of gluconeogenesis between 60 and 120 min was 63 μmoles/10 g.

domized in glucose (C. M. Veneziale, F. Gabrielli, and H. A. Lardy, unpublished data). Part of the randomization can be rationalized by invoking malic enzyme activity which has been shown (Shrago *et al.*, 1963) to supply pyruvate from malate at a rate sufficient to give significant randomization of pyruvate carbon during the relatively lengthy period of the perfusion. Pyruvate-*2*-^{14}C is converted into oxaloacetate under the influence of pyruvate carboxylase (Utter and Keech, 1963). Randomization of ^{14}C between the two halves of the dicarboxylic acids occurs, yielding malate-*2*-^{14}C and malate-*3*-^{14}C. From the latter, malic enzyme could catalyze the formation of pyruvate-*3*-^{14}C. This scheme would in effect lead to the production of pyruvate-*3*-^{14}C from pyruvate-*2*-^{14}C. Indeed, because of this argument, the pattern of ^{14}C labeling in glucose which was observed by others (Topper and Hastings, 1949) is not fully acceptable as proof that oxaloacetate must be converted into phosphoenolpyruvate in the conversion of pyruvate into glucose.

Discussion

The data of this paper can be interpreted to mean that the isolated rat liver can synthesize 3-phosphoenolpyruvate from pyruvate by a pathway(s) which does not require phosphoenolpyruvate carboxykinase. This working hypothesis has as its basis the observations that our liver preparations: (1) maintain a significant capability for converting pyruvate into glucose despite treatment with quinolinate; (2) accumulate large amounts of phosphoenolpyruvate, 2-phosphoglycerate, and 3-phosphoglycerate within 5 min after pyruvate is added to the perfusate, without concomitant accumulation of aspartate and malate; and (3) can incorporate isotope into 3-phosphoglycerate to the extent that its specific activity is greater than that of aspartate or malate within at least the first 10 min of perfusion with pyruvate-^{14}C. Hypothetical pathways to be considered are (1) direct conversion of pyruvate into phosphoenolpyruvate, (2) conversion of pyruvate into 3-phosphoglycerate without phosphoenolpyruvate being an obligatory intermediate, and (3) conversion of oxaloacetate into 3-phosphoglycerate without phosphoenolpyruvate being an obligatory intermediate.

The demonstration of two phosphoenolpyruvate pools, one which is glycolytic and a second which is glucogenic, rapidly turned over, and therefore heavily labeled with isotope from pyruvate-^{14}C, would support hypothetical pathway 1. The compartmentation of phosphoenolpyruvate in such a fashion, taken with the data of this paper, could be interpreted to mean that the isolated liver can synthesize phosphoenolpyruvate from pyruvate directly without the four-carbon dicarboxylic acids being obligatory intermediates. However, we have been unable to obtain direct evidence for the compartmentation of phosphoenolpyruvate. An aqueous extract of a liver perfused for 10 min with oxaloacetate-*methylene*-^{14}C was found to have a high concentration of highly radioactive phosphoenolpyruvate. This finding argues against compartmentation, such as by the sequestration of phosphoenolpyruvate within a protein fraction.

Much consideration has been given in the literature to the possibility that phosphoenolpyruvate can be formed directly from pyruvate by a reversal of the pyruvate kinase step, although little emphasis has been placed on it in recent years. Experimental data obtained for liver tissue suggest that the direct pathway does occur (Topper and Hastings, 1949; Lorber *et al.*, 1950; Hoberman and D'Adamo, 1960a,e; Bloom and Foster, 1962; Shreeve and De Meutter, 1964). We have no evidence that the reversal of the pyruvate kinase step is the mechanism for phosphoenolpyruvate formation from pyruvate in our liver preparations. By using the $\Delta F°$ of Burton and Krebs (1953) for the pyruvate kinase reaction and the 65-min data of Table III (expt 376) and assuming a ratio of ATP:ADP of 4.6 (Haeckel and Haeckel, 1968), the calculated free-energy change is such as to make phosphoenolpyruvate synthesis by this route most unlikely. Another hypothetical direct route would be that of phosphoenolpyruvate synthetase (Cooper and Kornberg, 1965) which occurs in *Escherichia coli* but has not been demonstrated in mammalian tissues.

We have attempted to detect the conversion of pyruvate into 3-phosphoglycerate in rat liver supernatant but without success. We also have looked for the possible hydroxylation of pyruvate by the microsomal fraction and as yet have no evidence for this reaction either.

With respect to an evaluation of hypothetical route 3, we have been unable to determine the specific activity of oxaloacetate. Without a precise determination of this parameter we can only speculate on the role of oxaloacetate in gluconeogenesis from pyruvate-^{14}C in the experiments of Tables IV and V. We assume that relatively rapid equilibration between malate and oxaloacetate occurred in our liver preparations and that it would be achieved within 3 to 5 min, based on the observation that 10 min was required to achieve equilibrium between oxaloacetate and aspartate. Arguments that oxaloacetate and malate can equilibrate more rapidly than oxaloacetate and aspartate are that (1) malate achieved a relatively high specific activity as early as 63 min (Table IV) and this very well may have been near the maximum that was achieved in the absence of caprylate, (2) the malate pool was much smaller than the aspartate pool, and (3) the only major source of oxaloacetate excluding the pyruvate carboxylase step is malate itself. Hypothetical pathway 3 would

explain the data of Table V but it requires that we postulate two oxaloacetate pools, one of which fails to equilibrate with malate and which would have to give rise to 3-phosphoglycerate by reactions which do not involve phosphoenolpyruvate. Although we have no direct evidence for this speculation, an evaluation of it might result from our isolation of two unidentified metabolites which become labeled in livers perfused with pyruvate-^{14}C or with pyruvate and bicarbonate-^{14}C. The labeling appears to increase under some conditions of augmented gluconeogenesis and we are now attempting to isolate sufficient quantity to permit identification.

If, as it is claimed, the quinolinate block is so highly effective, why was the conversion of alanine-*U*-^{14}C into glucose in the presence of inhibitor as much as one-third that in its absence (Table II)? Despite a 90% inhibition in the rate of gluconeogenesis, there was much less inhibition of alanine-*U*-^{14}C utilization for gluconeogenesis or as carbon source for glucose. This means that a selective utilization of alanine-^{14}C for gluconeogenesis occurred, perhaps by a more direct conversion of pyruvate-*U*-^{14}C (derived from alanine-*U*-^{14}C) into glucose than that requiring phosphoenolpyruvate carboxykinase activity. As does the apparently selective utilization of pyruvate-*3*-^{14}C (Table IV) for gluconeogenesis in the presence of quinolinate, that of alanine-*U*-^{14}C (Table II) argues favorably for a highly effective *in vivo* inhibition of phosphoenolpyruvate carboxykinase activity by quinolinate.

Previous work has shown that quinolinate inhibits gluconeogenesis completely when 20 mM alanine is used as substrate (Veneziale *et al.*, 1967). Why was the pyruvate derived from that substrate not also converted into glucose by one of the proposed hypothetical pathways suggested by the data of this paper? Very likely, the high amino nitrogen concentration itself also contributed to the inhibition of gluconeogenesis by adversely influencing a pathway of gluconeogenesis which does not require phosphoenolpyruvate carboxykinase, such as for serine (C. M. Veneziale, F. Gaflrielli, and H. A. Lardy, manuscript in preparation): for example, livers perfused with quinolinate and 2.0 mM serine can produce more glucose than livers perfused with quinolinate and 4.0 mM serine. A plausible explanation is that the excess amino nitrogen in some way interferes with the net consumption of the amino acid pool. This explanation is supported by the fact that rates of gluconeogenesis with quinolinate and 4.0 mM serine can be restored by addition of glucagon, 0.1 mM pyridoxal, or 1.0 mM pyruvate to the perfusate.

The data of Table V confirm the profound influence exerted by fatty acids, even at physiologic concentrations, on the metabolism of pyruvate in the fasted liver. It is possible that in the presence of fatty acids, any mechanisms that might exist for the metabolism of pyruvate by an alternative pathway become nonoperative or secondary. Most of the observations made at 65 min in the presence of added pyruvate and caprylate can be explained by a stimulation of the pyruvate carboxylase reaction and by an increase in flux from phosphoglycerate to glucose. Our concepts concerning the mechanisms by which these effects are brought about have already been presented in considerable detail (Veneziale *et al.*, 1967). That a favorable DPNH:DPN ratio is necessary for glucose biosynthesis was appreciated even before other important aspects of the process, as we know them now, were established. Evidence for the coupling of the DPNH formed by the glycerophosphate dehydrogenase system to the DPNH participating in glycogen synthesis was obtained by Bloom (1959) by means of liver and kidney slice experiments. Hoberman and D'Adamo gave evidence for the coupling of the oxidation of lactate (1960a,c) and indeed of malate (1960b) to the reductive synthesis of triosephosphate in livers of intact rats. Much has been written on the generation of extramitochondrial reducing power in gluconeogenesis (Krebs *et al.*, 1967) and on the specific influence of fatty acids on gluconeogenesis (Struck *et al.*, 1965; Williamson *et al.*, 1966; Veneziale *et al.*, 1967); additional discussion is not warranted here.

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Biochemical Characterization of Gastroferrin*

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ABSTRACT: Gastroferrin is a glycoprotein of human gastric juice which may be operative in the regulation of iron absorption. This protein has been purified and characterized using a variety of physical and chemical techniques. Although it migrates as a single peak in the analytical ultracentrifuge, it can be fractionated using DEAE-cellulose chromatography into several macromolecular species which appear to differ only in their sialic acid content. Gastroferrin binds 6% by weight of iron, and removal of sialic acid does not affect its iron binding capacity. The polypeptide portion of the glycoprotein, 15% by weight of the molecule, is rich in threonine, proline, and serine. The remaining 85% of the molecule is predominantly carbohydrate and consists of galactose, fucose and the acetyl derivatives of sialic acid, glucosamine, and galactosamine. Analytical ultracentrifugation gave a molecular weight of 2.63×10^5 and a sedimentation constant, $s^0_{20,w}$, of 8.9 S. Osmometry, however, yielded a slightly lower value of 2.35×10^5 for the number-average molecular weight indicating a small degree of microheterogeneity. Viscosity measurements demonstrated that gastroferrin was very asymmetric with an intrinsic viscosity, $[\eta]$, of 0.39 dl/g, and a frictional ratio of 2.2. Combination of viscosity data and sedimentation constant provided another independent estimate of molecular weight, 2.78×10^5, in good agreement with the value from sedimentation equilibrium. The immune behavior and iron binding of gastroferrin have been compared with those of soluble blood group substances from ovarian cyst fluid.

Equilibrium binding and chelation of ferric iron appear to be the primary factors in the regulation and control of the transport of iron by intestinal mucosa (Forth *et al.*, 1965; Hopping and Ruliffson, 1966; Helbock and Saltman, 1967; Spiro and Saltman, 1969). Both Fe^{2+} and Fe^{3+} are utilized when bound to a small molecular weight chelate. The amount of iron available for absorption is a function of the competition for dietary iron between the low molecular weight chelates and macromolecular ligands in the lumen of the small intestine. Only the former are utilized by the body for iron absorption. The role of endogenous ligands secreted into the digestive tract in man was studied by Davis *et al.* (1967), who found in gastric juice a high molecular weight component excluded by Sephadex G-200 and capable of binding all the iron present in a typical daily diet. This component was called gastroferrin. Of particular interest was their observation that gastroferrin was absent or low in several cases of iron storage associated with hemochromatosis (Davis *et al.*, 1966), suggesting that the amount of this high molecular weight ligand regulated the uptake of dietary iron. Further, gastroferrin was present in lower concentrations in patients with iron deficiency anemia (Luke *et al.*, 1967) where enhanced iron uptake is observed.

We have been concerned with the isolation and purification of gastroferrin together with its biochemical characterization and the nature of its iron binding. In a preliminary communication (Davis *et al.*, 1969), we reported a simple method for its isolation from human gastric juice. This method is based on the coprecipitation of iron gastroferrin with ferric hydroxide followed by removal of iron and molecular sieving

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