Gluconeogenesis in the Isolated Rat Liver. Studies with Bicarbonate-^{14}C*

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ABSTRACT: The metabolic fate of bicarbonate-^{14}C in perfused isolated liver from fasted intact rats was studied primarily in the presence of pyruvate (initial perfusate concentration, 2.0 mM). The perfusate consisted of bovine erythrocytes (hematocrit value, 30%) suspended in Krebs–Ringer–phosphate solution containing 3% (w/v) bovine albumin and was gassed with 100% O_2.

The specific radioactivity of various glucogenic intermediates was determined in extracts of liver. By sampling multiple livers at short intervals during the 6 min following addition of pyruvate and bicarbonate-^{14}C to the perfusate, the following observations were made. (1) Specific radioactivity of malate increased rapidly during the first few minutes, became maximal at approximately 3 min, and thereafter tended to diminish. (2) Specific radioactivity of 3-phosphoglycerate, which tended to be less than that of malate during the first 3 min, increased steadily from 0 to 5 min; by 4 min it exceeded that of malate. (3) Specific radioactivity of phosphoenolpyruvate failed to increase after the second minute. The mean ratio of specific radioactivities of phosphoenolpyruvate to 3-phosphoglycerate during the 6-min interval was 0.28 (SD = 0.08; N = 13). (4) Specific radioactivity of aspartate was always less than that of 3-phosphoglycerate; mean ratio, aspartate to 3-phosphoglycerate, during the 6-min interval was 0.36 (SD = 0.08; N = 13). (5) At no time was the specific radioactivity of malate twice the maximal specific radioactivity of 3-phosphoglycerate. These observations fail to support the concept that gluconeogenesis from pyruvate and bicarbonate can occur only by means of puruvate carboxylase, malate dehydrogenase or glutamate–oxalacetate transaminase, and phosphoenolpyruvate carboxykinase acting in consonance with the mitochondria–cytosol relationship as it has so far been developed. Alternative explanations are discussed.

Data have been presented (Veneziale *et al.*, 1970) which suggest that rat liver can synthesize 3-phosphoglycerate from pyruvate by a pathway which does not require phosphoenolpyruvate carboxykinase activity. The crucial finding was that the specific radioactivity of 3-phosphoglycerate was greater than that of malate in liver actively synthesizing glucose from pyruvate-*3*-^{14}C. Although these observations could be explained by compartmentation phenomena within the cell, such phenomena have not been demonstrated.

During the formation of glucose from pyruvate and bicarbonate-^{14}C by means of pyruvate carboxylase, malate dehydrogenase or glutamate–oxalacetate transaminase, and phosphoenolpyruvate carboxykinase, as it has been stated to occur (see p 425 of review by Exton *et al.*, 1970), the cell should achieve a specific radioactivity of phosphoenolpyruvate greater than or equivalent to that of 3-phosphoglycerate. Furthermore, the specific radioactivity of phosphoenolpyruvate should be no greater than one-half that of the malate from which the cell forms it, using oxalacetate as an intermediate. The randomization of bicarbonate carbon within the four-carbon dicarboxylic acids (Solomon *et al.*, 1941; Hoberman and D'Adamo, 1960; Bloom and Foster, 1962; Haynes, 1965) explains the retention of no more than half the label in the phosphoenolpyruvate carboxykinase reaction.

The primary purpose of this study was to determine the relative incorporation, in isolated rat liver, of label from bicarbonate-^{14}C in the presence of pyruvate (2.0 mM) into intracellular malate, aspartate, phosphoenolpyruvate, and 3-phosphoglycerate. During the course of the perfusions, tissue specimens (no more than two from one liver) were taken at intervals during the 6 min after addition of pyruvate and bicarbonate-^{14}C to the perfusate and analyzed for the radioactivity in various glucogenic intermediates as a way of monitoring hepatic utilization of bicarbonate-^{14}C.

Materials and Methods

The livers used in the perfusion experiments were taken from fasted (24 hr), male, Sprague–Dawley rats weighing 400–500 g. The perfusate consisted of washed bovine erythrocytes suspended (hematocrit value, 30%) in Krebs–Ringer–phosphate solution (Umbreit *et al.*, 1964) containing 3% (w/v) bovine albumin (Sigma, fraction V powder). The perfusion method, liver sampling techniques, and assays for glucose and various intermediates have been described (Veneziale *et al.*, 1967). ATP, ADP, and AMP were determined by the methods of Adam (Bergmeyer, 1965). The flow of perfusate (100-ml volume) was maintained at approximately 12 ml/min per 10 g (wet wt) of liver. Substrates were added in 200-μmole quantities to give a final perfusate concentration of 2.0 mM. Either 1.5 or 160 μmoles of bicarbonate was added (160 μmoles in each bicarbonate-^{14}C experiment), giving exogenous perfusate bicarbonate concentrations of 0.015 or 1.6 mM, respectively. Bicarbonate and, when used, pyruvate were added at 60-min perfusion. The concentrations given ignore the contribution made by endogenous bicarbonate. In experiments in which the lesser amount of bicarbonate was added, the endogenous intrahepatic blood was collected and dis-

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TABLE I: Acid–Base Balance of Perfusate Entering and Leaving Livers.[a]

Pyruvate (mM)	0	2.0	2.0
$NaHCO_3$ (mM)	0.015	0.015	1.6
pH			
Arterial	6.84	6.81	6.84
Venous	6.80	6.77	6.80
P_{O_2} (mm Hg)			
Arterial	124	148	171
Venous	63	73	58
ΔP_{O_2}, A-V (mm Hg)	61	75	113
ΔP_{CO_2}, V-A (mm Hg)	3.7	6.0	8.5
Liver wt (g)	13	14	14

[a] Inflow and outflow blood samples were collected by means of needle and syringe through rubber tubing leading to and from livers at 2–4 min after pyruvate and bicarbonate additions to perfusate. Syringes were sealed immediately and put on ice; their contents analyzed within minutes. At the start of the perfusion the endogenous blood was collected and discarded in low-bicarbonate perfusions. Pyruvate and bicarbonate were added at 60 min. The perfusate bicarbonate values given do not take into account the endogenous bicarbonate.

carded. The times of all additions to the perfusate and of liver sampling are given in the appropriate legends.

Only reagent grade substrates and quinolinic acid were used. The quinolinic acid was purified by multiple recrystallizations from 40% acetic acid followed by complete removal of the solvent. Sodium bicarbonate-^{14}C was obtained from Cal Atomic (Los Angeles, Calif.), pyruvate-2-^{14}C from Amersham–Searle, and crystalline glucagon from Mann Research Laboratories. The glucagon was added to the perfusate as a suspension in 0.15 M NaCl.

Paper chromatographic methods alone or combined with high-voltage electrophoresis were utilized to separate the ^{14}C-labeled compounds contained in aqueous, neutralized, perchloric acid extracts of the perfused liver. In earlier experiments, 150 μl of extract was applied to the origin (in 5-μl portions). In later experiments, the equivalent of 500 μl of extract was quantitatively applied to the origin (500 μl of extract was evaporated to approximately 50 μl by a stream of nitrogen and this concentrated extract was applied in 5-μl portions; the transfer was completed with two 30- to 40-μl water washes).

Chromatography systems 1 and 2 have been described (Veneziale and Gabrielli, 1969). System 1E involved the application of 2000 V for 75 min (Gilson Electrophorator, Model D) to chromatograms that had been developed in system 1 solvents; a pyridine–acetate buffer (pH 3.0; 2.0 M in acetate) was utilized. The origin was placed in the cathode compartment. System 1 was used for the isolation of malate and phosphoenolpyruvate, system 2 or 1E for glucose, and system 1E for lactate and 3-phosphoglycerate. The individual species were located by radioautography, and the appropriate areas of the paper were excised and counted with an efficiency of 70% in a Nuclear-Chicago Mark II liquid scintillation counter. The scintillation liquid consisted of 0.1 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)] and 4.0 g of 2,5-diphenyloxazole made to 1 l. with toluene. To determine the radioactivity due to aspartate, the combined radioactivity due to aspartate and glucose was determined by system 1 (which fails to separate those two species) and the radioactivity due to glucose, as found by system 2 or 1E, was then substracted.

Radioactivity measurements of the intermediates in the tissue extracts were related to their chemical concentrations to yield data in units of disintegrations per minute per micromole. This method for determining the specific radioactivity of a compound depends on a high percentage recovery of that compound after its chromatographic separation. Recovery is believed to have been adequate in these experiments: mean recoveries (±SD; N) of ^{14}C-labeled standards utilizing system 1 were malate, 87% (±3; 6); aspartate, 91% (±5; 6); phosphoenolpyruvate, 75% (±2; 3); and 3-phosphoglycerate, 96% (±5; 6). The radioactivity counting was done within several days after the paper was immersed in scintillation liquid. It has been found that, once immersed, the paper which was the site to which phosphoenolpyruvate had migrated lost approximately 8% of its counts in 1 week.

It is possible that the recovery of standard phosphoenolpyruvate was calculated to be only 75% because of the presence of a labeled contaminant in our standard. In any case, even if the specific radioactivity (disintegrations per minute per micromole) of phosphoenolpyruvate were falsely low by 25% in the bicarbonate-^{14}C experiments presented here, the conclusions drawn from those experiments would remain applicable.

The method of Astrup *et al.* (1960) was used to evaluate the acid–base balance of perfusate entering and leaving the livers.

Results

The acid–base balance of perfusate entering and leaving livers (Table I) was evaluated because of the unusual ionic composition of the perfusate relative to that of mammalian extracellular fluid. Qualitatively, the unphysiologic features of the perfusate resemble those found in the blood of patients suffering from diabetic acidosis but are more extreme. Oxygen-carrying capacity of the perfusate was adequate as indicated by the partial pressure of oxygen.

Influence of Low Perfusate Bicarbonate on Hepatic Metabolites and Rates of Gluconeogenesis. The addition of 1.5 or 160 μmoles of bicarbonate had no significant effect on metabolite concentrations (pyruvate, phosphoenolpyruvate, 2-phosphoglycerate, and 3-phosphoglycerate) or rate of gluconeogenesis (in the first 15 min after addition: without added pyruvate, 23 μmoles/10 g wet wt (SD, 2; N = 3); with 200 μmoles of pyruvate, 41 μmoles (SD, 9; N = 6). It is probably impossible to decrease the intracellular bicarbonate concentration enough, in the system used, to limit the rate of formation of glucose from pyruvate.

At 5 min after addition of 160 μmoles of bicarbonate and 200 μmoles of pyruvate, means (±SD; N = 8) were: ATP, 2.34 ± 0.24 μmoles/g wet wt; ADP, 0.98 ± 0.17; AMP, 0.20 ± 0.02. By addition of internal ATP standards to our reacting mixes, it was determined that only about 80% of the ATP present in the tissue extracts was measured. Therefore, the true ATP/ADP was approximately 3. Nevertheless, the uncorrected ratios compare favorably to those found by others under different conditions (Exton and Park, 1969; Veech *et al.*, 1970).

Incorporation of Pyruvate-2-^{14}C into Glucose and Hepatic Metabolites during Low-Bicarbonate Perfusion. As in isolated liver perfused with pyruvate-3-^{14}C under more physiologic conditions (Veneziale *et al.*, 1970), the specific radioactivity

TABLE II: Incorporation of Pyruvate-2-^{14}C into Glucose and Gluconeogenic Metabolites by Isolated Liver.[a]

Experiment	A		B	
HCO_3^- added (μmoles)	1.5		160	
Perfusion with pyruvate-2-^{14}C and HCO_3^- (min)	5	10	5	10
^{14}C in perfusate glucose (dpm)[b]	7.1×10^6		6.6×10^6	
Metabolites (dpm/μmole)				
Lactate	160,000	130,000	93,000	150,000
Malate	31,000	43,000	35,000	63,000
Aspartate	14,000	43,000	12,000	30,000
Glutamate	61,000	89,000	55,000	97,000
Phosphoenolpyruvate	71,000	90,000	72,000	87,000
3-Phosphoglycerate	88,000	98,000	87,000	110,000

[a] Pyruvate and bicarbonate added at 60 min. Livers were sampled at 65 and 70 min. Carefully placed ligatures applied to each after the initial samplings prevented bleeding. In A and B, pyruvate-2-^{14}C was added to final perfusate concentration of 2.0 mM, 190,000 dpm/μmole. [b] Corrected to 10 g of liver. See Table III for the method used to estimate disintegration per minute in perfusate glucose.

TABLE III: Influence of Substrate on Incorporation of ^{14}C from Bicarbonate-^{14}C into Perfusate Glucose by Isolated Rat Liver in 5 min.[a]

	Without Quinolinate		With Quinolinate[c]	
Substrate[b]	Expt	^{14}C in Glucose[d]	Expt	^{14}C in Glucose[d]
L-Lactate	1A	4.4	1B	0.51
Pyruvate + C_8[e]	2A	4.1	2B	1.1
Pyruvate + G[f]	3A	2.7	3B	1.2
Pyruvate[g]	4A-1		4B	0.68
	4A-2	1.4		
	4A-3	1.3		
	4A-4	1.4		
	4A-5	1.3		
L-Serine[g]	5A-1	0.72		
	5A-2	0.60		
L-Alanine[g]	6A-1	0.51		
	6A-2	0.59		
None[g]	7A-1	0.47		
	7A-2	0.55	7B	0.60
	7A-3	0.61		

[a] Livers were sampled at 5 min after addition of 50 μCi of bicarbonate-^{14}C (7.2×10^5 dpm/μmole; 160 μmoles) and substrate (perfusate concentration, 2.0 mM). Additions were made after 60-min perfusion (55 min for alanine and serine). Perchloric acid extracts of liver samples were paper chromatographed in system 2, which separates glucose from all known intermediates of gluconeogenesis. Glucose spot was located by autoradiography and its radioactivity was measured by scintillation counting. From this and the concentration of glucose in the extract, disintegrations per minute per micromole was calculated. Radioactivity in perfusate glucose (disintegrations per minute) was estimated by multiplying total perfusate glucose concentration by calculated disintegrations per minute per micromole. On comparison of this method to osazone method for measuring disintegrations per minute in perfusate glucose, in seven experiments after 60-min perfusion with labeled serine, alanine, or pyruvate, the mean difference was 7.1% (SD = 3.9). [b] In addition to bicarbonate-^{14}C. [c] Quinolinate added at 30 min; perfusate concentration, 2.4 mM. [d] As dpm $\times 10^{-6}$ in perfusate glucose, corrected to 10 g wet wt of liver. [e] At 60 min, 0.18 mmole of caprylate was added. [f] At 60 min, 5 μg of glucagon was added. [g] Results of replicate experiments are shown.

of 3-phosphoglycerate exceeded that of malate and aspartate (Table II). This is inconsistent with the hypothesis that malate and aspartate, newly formed from intramitochondrial oxalacetate, enter the cytosol where they serve as the major carbon sources for 3-phosphoglycerate synthesis.

The extent of glucose labeling was not significantly increased with the greater amount of added bicarbonate. Table II also shows that labeling in malate, aspartate, glutamate, phosphoenolpyruvate, and 3-phosphoglycerate was comparable at both concentrations of exogenous bicarbonate. This indicates that the metabolism of pyruvate was not limited by the amounts of bicarbonate added.

Influence of Substrates on Incorporation of ^{14}C from Bicarbonate into Glucose. At 5 min after addition of bicarbonate-^{14}C, incorporation of ^{14}C into perfusate glucose was stimulated more by lactate than by pyruvate relative to control expt 7A (Table III). Neither alanine nor serine influenced utilization to any great extent. The inhibition of phosphoenolpyruvate carboxykinase by quinolinate (Veneziale *et al.*, 1967), which was considerable if not complete in expt 1–4, failed to diminish the incorporation of label into glucose in expt 7B. How could glucose-^{14}C be formed from bicarbonate-^{14}C if phosphoenolpyruvate carboxykinase were greatly inhibited? This is at least theoretically possible because serine-^{14}C can be formed from glycine-^{14}C (Meister, 1965) which can be formed from bicarbonate-^{14}C (Motokawa and Kikuchi, 1969). Subsequently, gluconeogenesis from serine-^{14}C could occur even when phosphoenolpyruvate carboxykinase is inhibited by quinolinate (Veneziale *et al.*, 1969). It remains to be determined if the isolated organ can utilize bicarbonate in this fashion.

The consistency of the data—of the 4A, 5A, 6A, and 7A series—indicates that the amount of added bicarbonate-^{14}C that was swept into the atmosphere as CO_2-^{14}C was consistent from one perfusion to the next. In any case, this is unimportant with respect to the metabolite-labeling data to be presented below because the primary interest there is in the labeling of each metabolite relative to that of the others and this does not depend on the amount of added bicarbonate-^{14}C lost from the perfusion system.

Comparison of the data of Table IV with those of Table III shows that, regardless of the perfusion condition investi-

TABLE IV: Influence of Substrate on Incorporation of ^{14}C from Bicarbonate-^{14}C into Perfusate Glucose by Isolated Rat Liver in 15 min.[a]

Substrate[b]	Without Quinolinate			With Quinolinate[c]		
	Expt	^{14}C in Perfusate Glucose[d]	Glucose Formed[e]	Expt	^{14}C in Perfusate Glucose[d]	Glucose Formed[e]
Pyruvate + G[f]	8A	.2	50			
Pyruvate[g]	9A-1	0		9B	1.3	13
	9A-2	9	46			
L-Alanine	10A	2	15	10B	0.75	11
L-Serine	11A	75	24	11B	0.56	15
None	12A	8	20	12B	0.74	16

[a] Conditions and methods as in Table III. [b] In addition to bicarbonate-^{14}C. [c] Quinolinate added at 30 min; perfusate concentration, 2.4 mM. [d] As dpm $\times 10^{-6}$ in perfusate glucose corrected to 10 g wet wt of liver. [e] In μmoles/15 min corrected to 10 g wet wt of liver. [f] At 60 min, 5 μg of glucagon was added. [g] Results of replicate experiments are shown.

TABLE V: Incorporation of ^{14}C from Bicarbonate-^{14}C into Gluconeogenic Intermediates by Isolated Rat Liver in 5 min.[a]

Substrate[b]	Quinolinate[c] (mM)	Expt	Malate		Aspartate		Phosphoenolpyruvate		3-Phosphoglycerate	
			dpm/μmole	μmole/g	dpm/μmole	μmole/g	dpm/μmole	μmole/g	dpm/μmole	μmole/g
L-Lactate	0	1A	48,000	0.070	10,000	0.42	NV[d]	0.14	20,000	0.22
	2.4	1B	54,000	0.57	19,000	0.66	NV	0.056	NV	0.03
Pyruvate + C_8[e]	0	2A	96,000	0.21	39,000	0.37	13,000	0.28	37,000	0.49
	2.4	2B	130,000	1.81	80,000	1.07	18,000	0.27	55,000	0.50
Pyruvate + G[f]	0	3A	40,000	0.13	18,000	0.56	NV	0.26	19,000	0.55
	2.4	3B	62,000	0.54	32,000	0.93	NV	0.22	39,000	0.42
Pyruvate[g]	0	4A-1	29,000	0.091	3,500	0.57	6,500	0.57	32,000	0.89
	0	4A-2	19,000	0.10	9,500	0.52	13,000	0.49	38,000	0.99
	2.4	4B	61,000	0.50	21,000	0.95	6,000	0.40	35,000	0.66
	0	4A′[h]	44,000	0.079	15,000	0.45	18,000	0.48	45,000	0.82
L-Serine[g]	0	5A-1	NV	0.049	4,100	0.37	7,400	0.14	24,000	0.25
	0	5A-2	10,000	0.081	7,400	0.42	8,700	0.085	28,000	0.22
L-Alanine[g]	0	6A-1	29,000	0.052	7,700	0.48	NV	0.17	21,000	0.31
	0	6A-2	10,000	0.079	11,000	0.39	4,000	0.17	18,000	0.40
None[g]	0	7A-1	NV	0.062	3,300	0.45	NV	0.26	12,000	0.45
	0	7A-2	21,000	0.035	3,600	0.39	6,100	0.12	18,000	0.27
	0	7A-3	9,300	0.079	5,500	0.37	7,000	0.12	26,000	0.26
	2.4	7B	69,000	0.20	34,000	0.74	NV	0.11	36,000	0.14

[a] Conditions and methods as in Table III. [b] In addition to bicarbonate-^{14}C. [c] Added at 30 min. [d] NV = not detectable by autoradiography. [e] At 60 min, 0.18 mmole of caprylate was added. [f] At 60 min, 5 μg of glucagon was added. [g] Replicate studies. [h] In this experiment, 100 μCi of bicarbonate-^{14}C was added. At 70 min, specific radioactivities were: malate, 25,000 dpm/μmole; aspartate, 11,000 dpm/μmole; phosphoenolpyruvate, 14,000 dpm/μmole; and 3-phosphoglycerate, 39,000 dpm/μmole.

gated, the rate of incorporation of bicarbonate-^{14}C into glucose was greater during the first 5 min than during the interval between 5 and 15 min.

Labeling of Malate, Aspartate, Phosphoenolpyruvate, and 3-Phosphoglycerate from Bicarbonate-^{14}C in the Presence and Absence of Quinolinate. In expt 4A-1 and 4A′ in Table V, the specific radioactivity of phosphoenolpyruvate was less than half that of malate. This is consistent with phosphoenolpyruvate formation from pyruvate as a result of the action of pyruvate carboxylase, malate dehydrogenase, and phosphoenolpyruvate carboxykinase (although it must be considered that some of the labeled phosphoenolpyruvate could have been formed as a result of enolase activity). However, in these experiments the specific radioactivity of 3-phosphoglycerate was significantly greater than that of phosphoenolpyruvate. Indeed, in each of the 11 experiments in which the specific radioactivity of phosphoenolpyruvate could be measured, it was less than half that of 3-phosphoglycerate. These observations contradict the concept that gluconeogenesis from pyruvate requires the conversion of phosphoenolpyruvate into 3-phosphoglycerate because, barring compartmentation of phosphoenolpyruvate and considering differences in pool sizes, precursor must be more labeled than product.

Caprylate profoundly stimulated malate-^{14}C and aspartate-

TABLE VI: Incorporation of ^{14}C into Gluconeogenic Intermediates and Glucose during Short-Term Perfusion with Pyruvate and Bicarbonate-^{14}C.[a]

	Study 1					Study 2				Study 3			
Liver[b] (min)	139-1 1	140-1 2	115 3½	139-2 5	140-2 6	141-1 1	142-1 2	141-2 4	142-2 5	145-1 2	146-1 2	146-2 4	145-2 5
Glutamate													
dpm/μmole	6,800	38,000	25,000	44,000	43,000	4,600	26,000	34,000	43,000	12,000	20,000	39,000	28,000
μmole/g	0.64	0.52	0.78	0.64	0.68	0.94	0.82	1.04	0.88	0.84	0.95	0.89	0.63
Malate													
dpm/μmole	24,000	30,000	34,000	27,000	16,000	18,000	37,000	20,000	17,000	14,000	37,000	27,000	21,000
μmole/g	0.098	0.092	0.079	0.089	0.087	0.098	0.11	0.11	0.10	0.10	0.10	0.12	0.073
Aspartate													
dpm/μmole	2,700	10,000	8,000	7,800	12,000	3,700	5,800	8,000	14,000	7,300	9,400	13,000	11,000
μmole/g	0.47	0.42	0.50	0.44	0.45	0.38	0.35	0.43	0.33	0.31	0.44	0.41	0.36
Phosphoenolpyruvate													
dpm/μmole	2,400	5,800	3,900	6,600	6,100	3,000	11,000	7,000	11,000	5,200	8,100	9,500	7,900
μmole/g	0.27	0.39	0.54	0.68	0.75	0.21	0.26	0.56	0.50	0.25	0.24	0.53	0.44
3-Phosphoglycerate													
dpm/μmole	8,500	30,000	23,000	33,000	29,000	6,800	26,000	25,000	34,000	16,000	28,000	36,000	30,000
μmole/g	0.45	0.74	0.92	1.21	1.39	0.41	0.58	1.02	1.04	0.50	0.49	1.02	0.79
Perfusate glucose[c]													
dpm × 10^{-6}	0.054	0.72	0.52	1.3[d]	1.1[d]	0.077	0.68	1.4[d]	1.4[d]	0.34	0.67	1.1[d]	1.3[d]

[a] Conditions and methods as in Table III. Samplings were at intervals shown in headings. [b] Number after hyphen indicates first or second specimen from that liver. [c] Corrected to 10 g wet wt of liver. [d] Falsely low by about 10% as calculation did not take into account the weight of ischemic tissue distal to ligature placed after first sampling.

^{14}C formation (expt 2A) but without increasing the label in phosphoenolpyruvate. The effects of glucagon on the incorporation of bicarbonate-^{14}C into hepatic metabolites (expt 3A) contrasted sharply with those due to caprylate, as was the case when pyruvate-*3*-^{14}C was the substrate (Veneziale *et al.*, 1970).

In all experiments of the B series—that is, those with quinolinate—there was an increase in isotope in the pools of malate and aspartate. This was secondary to an increase in pool sizes and to increases in specific radioactivities. The positive effect of quinolinate on the synthesis of labeled malate and aspartate was also demonstrated at 3.5 min after addition of pyruvate and bicarbonate-^{14}C to the perfusate (data not shown).

Labeling of Malate, Aspartate, Glutamate, Phosphoenolpyruvate, and 3-Phosphoglycerate from Bicarbonate-^{14}C in 0–6 Min. By sampling at different times within the 6 min after addition of pyruvate and bicarbonate-^{14}C (most livers were sampled twice), glucogenic events were monitored insofar as they are reflected by the specific radioactivities of the metabolites given in Table VI. The specific radioactivity of malate increased rapidly during the first few minutes, became maximal at approximately 3 min, and thereafter tended to decrease. The specific radioactivity of 3-phosphoglycerate increased until, at 4 or 5 min, it tended to exceed that of malate. In contrast, the specific radioactivity of phosphoenolpyruvate remained a relatively small fraction of that of 3-phosphoglycerate throughout most of the interval. At no time was the specific radioactivity of malate twice the maximal specific radioactivity achieved by 3-phosphoglycerate. The data of Table VI fail to support concepts of gluconeogenesis which do not allow for the possible existence of enzyme systems or compartmentation phenomena which remain to be elucidated. They can be interpreted to mean that intramitochondrial oxalacetate can serve as carbon source for phosphoglycerate by a pathway which does not include phosphoenolpyruvate.

Discussion

The metabolic sequence proposed (see p 425 of review by Exton *et al.*, 1970) for gluconeogenesis from pyruvate in the hepatic cell and catalyzed by pyruvate carboxylase, malate dehydrogenase or glutamate–oxalacetate transaminase, and phosphoenolpyruvate carboxykinase is characterized in the rat by the exclusive location of pyruvate carboxylase within the mitochondria (Walter *et al.*, 1966; Böttger *et al.*, 1969), from which egress of the product is restricted (Lardy *et al.*, 1965). In the present investigation, as well as in a preceding one (Veneziale *et al.*, 1970), an attempt was made to test these concepts by studying the metabolism of pyruvate-^{14}C and bicarbonate-^{14}C in isolated rat liver. The data obtained as a result of this approach indicate that gluconeogenesis from pyruvate might involve either (1) additional reactions, (2) additional compartmentation phenomena, or (3) both.

From the data in Table VI, in most liver specimens the specific radioactivity of phosphoenolpyruvate was less than half that of malate and therefore the oxalacetate of the cytosol must have been less labeled than malate or at least relatively poorly labeled at carbons-1, -2, and -3. In addition, the phosphoenolpyruvate carboxykinase reaction must have been slower than the process resulting in labeled 3-phosphoglycerate. The isotopic data of Table VI do not contradict this hypothesis. Intramitochondrial oxalacetate very likely became highly radioactive after addition of bicarbonate-^{14}C to the perfusate. This is supported by the relatively great labeling of malate and glutamate during the metabolism of pyruvate and bicarbonate-^{14}C (Table VI), which surely must re-

flect that of mitochondrial oxalacetate, a precursor of both compounds. The foregoing considerations, taken together with our current knowledge of the intracellular distribution of phosphoenolpyruvate, point to mitochondrial oxalacetate as the major carbon source of 3-phosphoglycerate by a pathway which does not appear to involve cytoplasmic phosphoenolpyruvate. This hypothesis is being evaluated by means of cell-free systems.

Another cause of the limited labeling of phosphoenolpyruvate might be that the hepatic cell of the fasted rat separates phosphoenolpyruvate into a labeled gluconeogenic pool and an unlabeled glycolytic pool. Analyses of tissue extracts would then yield an average specific radioactivity for that metabolite. Indeed, this must be reckoned with especially since enolase has been found in the nuceli of rat liver by Dounce (1950), and its presence there as well as in the cytosol suggests the possibility of two phosphoenolpyruvate pools. In preliminary experiments we have been able to demonstrate the conversion of phosphoenolpyruvate to 2-phosphoglycerate and of 2-phosphoglycerate to phosphoenolpyruvate by liver nuclei of fasted rats prepared by the method of Chauveau *et al.* (1956). However, we could not demonstrate the presence of endogenous phosphoenolpyruvate in two such nuclei preparations.

Attention is again called to the unphysiologic nature of the perfusate, which detracts from the physiologic significance of the data of this paper. However, there is no basis for believing that the hepatocyte can use pathways under these conditions that are not also operative under normal conditions. Furthermore, the pattern of metabolite labeling found in livers metabolizing labeled pyruvate (Table II) was similar to that found in livers metabolizing labeled pyruvate in a Krebs–Ringer–bicarbonate perfusate (Veneziale *et al.*, 1970).

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