The Effect of Variation of CO_2 Concentration on Pyruvate Metabolism *in Vitro**

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ABSTRACT: The effect of varying the HCO_3^- concentration of incubation medium from low (10 mM) to high (40 mM) physiological concentrations at pH 7.40 on pyruvate-2-^{14}C metabolism in rat liver slices has been studied. These studies were carried out both in the presence of a high concentration of pyruvate (37 mM) and a more nearly physiological concentration (0.3–0.5 mM). The conversion of pyruvate-2-^{14}C into glucose and glycogen was unaffected by variation in HCO_3^- concentration under these conditions. Pyruvate oxidation to CO_2 was increased (20–60%) by a rise in HCO_3^- concentration at both substrate concentrations as was the conversion of pyruvate into triglyceride and phospholipid fatty acids (100–216%). Pyruvate 2-^{14}C conversion into cholesterol was unaffected by a change in HCO_3^- concentration when pyruvate was present at 37 mM. However, when pyruvate concentration was reduced to the lower level, the conversion of pyruvate into cholesterol was increased (70–95%) by the increase in HCO_3^- concentration. The possible role of HCO_3^- concentration in regulating fatty acid and cholesterol synthesis is discussed.

It has previously been reported that acetate conversion into triglyceride and phospholipid fatty acids is increased in rat liver slices when the total CO_2 concentration of the medium is increased at a constant pH (Longmore *et al.*, 1967). No increase in acetate conversion into cholesterol was observed. Earlier studies under similar conditions reported an increased conversion of glucose and fructose into glycogen (Hastings and Longmore, 1965) and of glycerol into glycogen (Hastings *et al.*, 1962; Longmore *et al.*, 1964) with an increase in total CO_2 concentration. In view of the central role pyruvate plays in linking glycolysis, gluconeogenesis, fatty acid synthesis, and mitochondrial oxidation, and the involvement of CO_2 as either a reactant or product in many of these pathways, a study of the effect of variations in total CO_2 concentration on pyruvate-2-^{14}C conversion into glycogen, glucose, CO_2, fatty acids, and cholesterol was undertaken. It was hoped that these experiments, in which total CO_2 concentration of the media was varied within the physiological limits of extracellular fluid, and pyruvate concentration varied from above normal to physiological levels, would provide some insight into the possible role of cellular CO_2 concentration as a controlling factor in the metabolism of the liver cell.

Methods

Male rats of the Sprague–Dawley strain fed Purina laboratory chow, weighing 300–400 g and fasted for 16–18 hr before death, were used in these studies. Procedures for liver slicing, tissue incubation, as well as the cationic and anionic composition of the media used were as previously reported (Longmore *et al.*, 1964). Except for that amount of sodium pyruvate-2-^{14}C[1] (3 μCi/flask) used in the media preparation, pyruvate was present as the potassium salt. Following a 10-min preincubation of the tissue in substrate free media, the tissue was transferred to incubation flasks containing either 37 mM pyruvate-2-^{14}C alone, or 0.3–0.5 mM pyruvate-2-^{14}C and 30 mM glucose. Tissue incubation was for either a 60- or 90-min period as indicated in the tables.

In the series of experiments in which pyruvate was maintained between 0.46 and 0.39 mM during the incubation period, pyruvate-2-^{14}C was added by microsyringe through the rubber side port of the incubation flasks at 7-min intervals. The amounts required to maintain the desired pyruvate concentrations had been determined in preliminary experiments in which pyruvate utilization was followed during incubation. A 69.2 mM solution of pyruvate-2-^{14}C, adjusted to isotonicity with KCl, was added to the incubation flasks, containing 10 ml of media, at 7-min intervals, 24.4 μl at each interval to the low HCO_3^- medium and 34.0 μl at each interval to the high HCO_3^- medium. This solution of pyruvate-2-^{14}C was a portion of that used to prepare the media originally placed in the incubation flasks and thus was of identical specific activity.

Procedures for CO_2 determination, lipid extraction, separation into classes and quantitation, as well as counting procedures were the same as those previously reported (Longmore *et al.*, 1967). Determination of media pH was made using a microcapillary glass electrode unit. Medium pyruvate determination was per-

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[1] Obtained from New England Nuclear Corp.

TABLE I: Effect of Varying Medium HCO_3^- Concentration on the Conversion of Pyruvate-2-^{14}C into CO_2, Glucose, Glycogen, Triglyceride Fatty Acid (TGFA), Phospholipid Fatty Acid (PLFA), and Cholesterol.[a]

Experiment	pH	HCO_3^- (mM)	Pyruvate Uptake[b]	Pyruvate Converted into				
				CO_2[b]	Glucose[b]	Glycogen[b]	TGFA[b,c]	PLFA[b,c]	Cholesterol[b,c]
1a	7.40	39.7	213	67.0	25.4	9.8	9.5	7.7	1.8
1b	7.46	11.7	266	61.6	29.8	9.7	4.5	5.6	1.8
2a	7.40	38.3	241	51.5	24.3	3.2	6.0	5.4	2.6
2b	7.35	6.9	252	39.9	24.2	3.1	3.1	2.6	2.5
3a	7.40	38.9	270	67.2	21.3	5.5	5.0	6.0	1.0
3b	7.36	7.2	239	50.5	21.7	3.3	2.3	2.5	0.8
4a	7.38	40.7	246	62.5	10.9	0.6	4.0	4.6	1.5
4b	7.41	8.4	266	56.0	11.4	0.7	2.2	2.0	1.6
Average a	7.40	39.3	243	62.1	20.5	4.8	6.1	5.9	1.7
Average b	7.40	8.6	256	52.0	21.8	4.2	3.0	3.2	1.7
Average a:b ratio			0.95	1.20	0.94	1.14	2.03	1.84	1.0
Average range of triplicates	0.01	0.20	20	4.5	1.8	0.7	0.6	0.6	0.2

[a] *90-min incubation; initial pyruvate concentration, 37 mM.* [b] *μmoles/g per 90 min.* [c] $\times 10^{-1}$.

formed enzymatically using lactic dehydrogenase after perchloric acid treatment and neutralization. Pyruvate was isolated from the media as the 2,4-dinitrophenylhydrazone and radioactivity was determined by gas-flow counting.

After perchloric acid treatment and neutralization of the media, the glucose concentration of the media was determined by assay using glucose oxidase. An aliquot of the deproteinized medium was then placed on a Dowex 1 (Cl^-) column. The glucose fraction was collected during elution of the column with water. After concentration, the specific activity of this glucose was then determined by scintillation counting and quantitation of the glucose present by glucose oxidase. Following lipid extraction, the liver pellet was hydrolyzed in 30% KOH and an aliquot was taken for nitrogen determination by H_2SO_4–H_2O_2 digestion followed by Nesslerization in order to obtain the liver equivalent wet weight. Tissue glycogen content and counting procedures were as reported earlier (Longmore *et al.*, 1964).

Results

Each experiment cited represents the average data obtained from three sets of liver slices incubated in separate flasks. The average range of such triplicate determinations for all experiments is given in the last line of the tables. Below the data of the individual experiments in each table are presented the average values of the data from four or five experiments, average a representing data obtained at high HCO_3^- concentration and average b that obtained at low HCO_3^- concentration. The average a:b ratio is also given.

A comparison of the conversion of pyruvate-2-^{14}C present initially at 37 mM into CO_2, glucose, glycogen, triglyceride fatty acids, phospholipid fatty acids, and cholesterol at 8.7 and 39.3 mM HCO_3^- is shown in Table I. The average pH of the medium during incubation was 7.40 at both HCO_3^- concentrations. Pyruvate uptake and pyruvate conversion into glucose, glycogen, and cholesterol remained essentially unaffected by a change in HCO_3^- concentration. The oxidation of pyruvate-2-^{14}C to CO_2 was increased 20% and the conversion into triglyceride and phospholipid fatty acids was increased 103 and 84%, respectively, by the increase in HCO_3^- concentration.

The effect of variation of HCO_3^- concentration on pyruvate-2-^{14}C metabolism when pyruvate was present initially at 0.27 mM, a value near physiological concentration, is presented in Table II. The uptake of pyruvate and conversion into glycogen was unaffected by the change in HCO_3^- concentration. A decrease in pyruvate conversion into glucose (47%) was observed with the rise in HCO_3^- concentration from 6.3 to 37.8 mM at pH 7.40. An increase in pyruvate conversion into CO_2 (64%), triglyceride fatty acid (214%), phospholipid fatty acid (216%), and cholesterol (95%) was observed under these conditions. During this 60-min incubation, the pyruvate uptake averaged 81% of the total pyruvate present.

To minimize the effect of recycling pyruvate metabolites and to maintain a more nearly constant specific

activity of pyruvate over the 60-min incubation period, a series of experiments was performed in which pyruvate-2-^{14}C was added to the individual flasks in an amount equivalent to the predetermined utilization rate for each of the two HCO_3^- concentrations. Initial pyruvate concentration averaged (± std dev) 0.46 ± 0.01 mM and final pyruvate concentration averaged 0.39 ± 0.05 mM for all media. The results of these experiments are presented in Table III. With a rise in HCO_3^- concentration from 5.1 to 40.8 mM at pH 7.43 no change in pyruvate conversion into glucose or glycogen was observed. With an increase in HCO_3^- concentration, a 20% increase in pyruvate uptake was observed and pyruvate conversion into CO_2 and into cholesterol was increased 46 and 70%, respectively. Pyruvate conversion into triglyceride and phospholipid fatty acids was not measured in this series of experiments.

TABLE II: Effect of Varying Medium HCO_3^- Concentration on the Conversion of Pyruvate-2-^{14}C into CO_2, Glucose, Glycogen, Triglyceride Fatty Acid (TGFA), Phospholipid Fatty Acid (PLFA) and Cholesterol. 60 Minute Incubation.[a]

Experiment	pH	HCO_3^- (mM)	Pyr Uptake[b]	Pyruvate Converted into					
				CO_2[b]	Glucose[b,c]	Glycogen[b,c]	TGFA[b,d]	PLFA[b,d]	Cholesterol[b,d]
1a	7.41	37.1	8.5	6.9	1.5	1.0	4.4	2.7	4.2
1b	7.42	7.6	6.6	3.1	2.6	0.8	1.0	0.5	2.0
2a	7.42	36.8	7.6	6.4	3.5	1.6	10.1	6.7	17.3
2b	7.37	6.3	7.2	4.9	6.9	2.4	4.1	2.1	9.1
3a	7.40	36.2	5.9	4.5	2.6	1.1	6.1	2.9	9.1
3b	7.37	6.0	5.3	2.7	4.0	1.1	1.5	1.2	3.8
4a	7.42	41.0		3.8	0.9	1.3			20.3
4b	7.41	5.2		2.5	2.5	1.8			10.9
Average a	7.41	37.8	7.5	5.4	2.1	1.3	6.9	4.1	12.7
Average b	7.39	6.3	6.9	3.3	4.0	1.5	2.2	1.3	6.5
Average a : b ratio			1.09	1.64	0.53	0.87	3.14	3.16	1.95
Average range of triplicates	0.01	0.15	0.4	0.3	0.3	0.2	0.6	0.5	0.7

[a] Initial pyruvate concentration, 0.270 ± 0.001 mM (±std dev); initial glucose concentration, 30 mM. Concentration of pyruvate at termination of incubation averaged 0.047 ± 0.001 mM (± std dev). [b] μmoles/g 60 min. [c] $\times 10^{-1}$. [d] $\times 10^{-3}$.

Discussion

The results presented in the present communication indicate that unlike glucose, fructose (Hastings and Longmore, 1965), or glycerol (Longmore *et al.*, 1964), pyruvate conversion into glucose and glycogen is essentially unaffected by variations in HCO_3^- concentration within what might be considered physiological limits of extracellular HCO_3^- concentration. This is so both at saturating levels of pyruvate (37 mM) and at the more nearly physiological level of pyruvate, 0.3–0.4 mM, (Goodwin and Williams, 1952), with the exception that the apparent conversion of pyruvate into glucose decreased (47%) at the high HCO_3^- concentration when the lower pyruvate level was allowed to decrease further during incubation. A recent report (Dyson and Anderson, 1968) has indicated an inhibition of rat liver microsomal glucose-6-phosphatase activities by increased HCO_3^- concentration. While such an inhibition might explain the decreased conversion of pyruvate into glucose, it is not clear why this inhibition would be observed only at very low pyruvate concentrations in the experiments reported here. An increase in pyruvate oxidation to CO_2 was observed with an increase in medium HCO_3^- concentration under conditions of both high and physiological levels of pyruvate, but was greater at the physiological pyruvate concentration range. In the situation where pyruvate concentration was maintained within the physiological range during incubation, an increase in pyruvate uptake was observed with increased HCO_3^- concentration. Both increased uptake and oxidation to CO_2 had been shown to occur with increased HCO_3^- concentration with glycerol as substrate (Longmore *et al.*, 1964).

The increase in pyruvate conversion into triglyceride and phospholipid fatty acids with increased HCO_3^- concentration is similar to the data published concerning the effect of HCO_3^- concentration on acetate metabolism in liver slices (Longmore *et al.*, 1967). It is significant that this stimulation of fatty acid synthesis occurs even when pyruvate concentration is at physiological or lower levels. The earlier results on the HCO_3^- effect on acetate conversion into triglyceride and phospholipid fatty acids was performed only at high levels of acetate

TABLE III: Effect of Varying Medium HCO_3^- Concentration on the Conversion of Pyruvate-2-^{14}C into CO_2, Glucose, Glycogen, and Cholesterol.[a]

Experiment	pH	HCO_3^- (mM)	Pyruvate Uptake (μmoles/g 60 min)	Pyruvate Converted into			
				CO_2 (μmoles/g 60 min)	Glucose (μmoles/g 60 min) $\times 10^{-1}$	Glycogen (μmoles/g 60 min) $\times 10^{-1}$	Cholesterol (μmoles/g 60 min) $\times 10^{-1}$
1a	7.44	41.0	19.2	13.3	9.0	4.7	3.4
1b	7.43	5.2	14.3	6.8	6.8	3.0	2.2
2a	7.45	41.2	17.3	6.4	3.9	1.1	22.5
2b	7.42	4.8	14.4	4.2	5.0	1.5	11.6
3a	7.41	40.9	17.4	10.5	6.3	4.2	17.6
3b	7.39	5.0	15.8	9.2	6.8	4.1	10.6
4a	7.44	40.4	18.1	12.1	7.7	1.5	6.0
4b	7.43	5.7	17.6	9.7	10.9	3.1	5.7
5a	7.44	40.3	17.3	13.2			10.2
5b	7.40	5.1	13.1	8.2			5.1
Average a	7.44	40.8	17.9	11.1	6.7	2.9	11.9
Average b	7.42	5.1	15.0	7.6	7.4	2.9	7.0
Average a: b ratio			1.20	1.46	0.91	1.00	1.70
Average range of triplicates	0.01	0.17	1.1	1.2	0.9	0.6	1.5

[a] 60-min incubation; initial pyruvate concentration, 0.46 ± 0.01 mM (±std dev), and was maintained at this value by substrate addition; initial glucose concentration, 30 mM. Concentration of pyruvate at termination of incubation averaged 0.39 ± 0.05 mM (± std dev).

(10 mM). Thus, while factors such as citrate and long-chain acyl-*S*-CoA's[2] (Numa *et al.*, 1965) are thought to control fatty acid synthesis at the acetyl-CoA carboxylase step, the question of whether changes in extracellular HCO_3^- concentration may also regulate the rate of fatty acid synthesis at this step arises. Variation of HCO_3^- concentration through the physiological range has been shown to influence the synthesis of fatty acids in a cell-free system of rat liver (Doering and Tarver, 1965) as well as in rat liver slices (Longmore, 1966). Both the rate of fatty acid synthesis and the carbon number of the fatty acids synthesized were shown to be altered by changes in HCO_3^- concentration in a cell-free preparation of lactating rabbit mammary gland (Smith and Dils, 1966).

The conversion of pyruvate into cholesterol with a pyruvate concentration of 37 mM was unaffected by variations of HCO_3^- concentration, similar to the data previously obtained with acetate (Longmore *et al.*, 1967). However, when pyruvate concentration was reduced to the lower more physiological level, a striking increase in cholesterol synthesized from pyruvate was observed with increased HCO_3^- concentration. This increase was present regardless of whether the pyruvate concentration was allowed to decrease further during the incubation or maintained at a nearly constant level of 0.4 mM.

A pathway for β-hydroxy-β-methylglutaryl-CoA synthesis involving malonyl-CoA as an intermediate has been described in pigeon liver (Brodie *et al.*, 1963). However, Fimognari and Rodwell (Fimognari and Rodwell, 1964), using isolated rat liver enzyme systems, presented data which suggested that the pathway described by Brodie was of little significance in mammalian tissue when compared with the classical pathways for mevalonate and ultimately cholesterol biosynthesis (Rudney, 1957). A possible explanation for the increase in the pyruvate conversion into cholesterol with increased HCO_3^- concentration and low levels of pyruvate could be that of an increased significance of malonyl-CoA participation in β-hydroxy-β-methylglutaryl-CoA synthesis under these conditions. This would be expected if the K_m for acetyl-CoA in the carboxylase reaction was much lower than its K_m's in the classical pathway for β-hydroxy-β-methylglutaryl-CoA synthesis. Since both acetyl-CoA and pyruvate are present in quite low concentrations in the intact animal, the malonyl-CoA pathway for the synthesis of β-hydroxy-β-methylglutaryl-CoA may be of considerable importance.

This discussion of the effect of HCO_3^- on fatty acid and cholesterol synthesis is based upon the concept that the basic effect of increased HCO_3^- is to increase directly malonyl-CoA production. It is realized that many other explanations of the data are possible and the exact mechanism by which HCO_3^- exerts its effect remains to be determined.

[2] Abbreviation is as listed in *Biochemistry 5*, 1445 (1966).

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Induction of Phosphopyruvate Carboxylase in Neonatal Rat Liver by Adenosine 3′,5′-Cyclic Monophosphate*

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ABSTRACT: The activity of cytoplasmic phosphopyruvate carboxylase (guanosine triphosphate:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32) normally develops in liver following birth or premature delivery of the fetus. The enzyme activity can be precociously induced *in utero* by intraperitoneal injection of 3′,5′-cyclic adenosine monophosphate to fetuses, the effect is largely prevented by simultaneous injection of actinomycin D and approximately doubled by simultaneous caffeine injection. The postnatal development of enzyme activity demonstrable in prematurely delivered rats is increased by injection of 3′,5′-cyclic adenosine monophosphate and the effect is potentiated by caffeine. N^6-2′-*O*-Dibutyryl 3′,5′-cyclic adenosine monophosphate is more potent than the parent compound in enzyme induction in both fetuses and premature animals but 5′-adenosine monophosphate, 2′,3′-cyclic adenosine monophosphate, 3′,5′-cyclic thymidine monophosphate, 3′,5′-cyclic guanosine monophosphate, and 3′,5′-cyclic inosine monophosphate are ineffective. No effect of dibutyryl 3′,5′-cyclic adenosine monophosphate is found on fetal liver activities of fructose diphosphatase and pyruvate kinase. Repression of phosphopyruvate carboxylase synthesis in premature rat liver is achieved by injection of insulin, ergotamine tartrate, or prostaglandin PGE_1. Activation of hepatic phosphopyruvate carboxylase by 3′,5′-cyclic adenosine monophosphate *in vitro* could not be demonstrated. The results are discussed in terms of mechanisms of enzyme induction in tissue differentiation.

Previous work has shown that the activity of cytoplasmic phosphopyruvate carboxylase (GTP[1]:oxaloacetate carboxy-lyase transphosphorylating, EC 4.1.1.32) develops postnatally in the rat liver (Ballard and Hanson, 1967; Yeung *et al.*, 1967) and that premature delivery of fetuses by uterine section also results in rapid appearance of the enzyme (Yeung and Oliver, 1967). Administration of the glucocorticoid analog, triamcinolone, to fetal rats *in utero* does not lead to development of the enzyme (Yeung *et al.*, 1967). In a more recent study Yeung and Oliver (1968) have shown that the administration of glucagon, adrenalin, and noradrenalin to fetal rats results in development of the carboxylase activity *in utero*. In addition, glucagon injection to surgically delivered rats potentiates the postnatal production of the enzyme. As these hormones have been shown to promote the production of 3′,5′-cyclic AMP in liver (Sutherland and Robinson, 1966), Yeung and Oliver (1968) have suggested that the cyclic nucleotide may function in the system as an effector molecule bringing about derepression of the synthetic system for cytoplasmic phosphopyruvate carboxylase. In the present paper further evidence, both direct and indirect, is presented to support the hypothesis that 3′,5′-cyclic AMP is involved in the initiation of cyto-

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[1] Abbreviations are listed in *Biochemistry 5*, 1445 (1966).