The Effects of Energetic Steady State, Pyruvate Concentration, and Octanoyl-(-)-carnitine on the Relative Rates of Carboxylation and Decarboxylation of Pyruvate by Rat Liver Mitochondria*

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Rat liver mitochondria were incubated with controlled concentrations of pyruvate over a range of energetic and respiratory steady states, in the presence and absence of octanoyl-(-)-carnitine in order to evaluate conditions and effectors which perturb the absolute and relative rates of flux through pyruvate carboxylase and pyruvate dehydrogenase. Control experiments using saturating concentrations of pyruvate are also reported.

With high [pyruvate] (10 mM) as sole substrate, carboxylation rate was very rapid in the resting state, and was diminished in a stepwise manner on stimulation of respiration with increasing amounts of ATPase. Carboxylase flux rates did not correlate with [acetyl-CoA] or acetyl-CoA/CoASH ratio, or with changes in the mitochondrial ATP/ADP until ATPase in excess of maximal respiratory stimulation was added. Octanoyl carnitine stimulated carboxylation further, and this rate was better sustained in stimulated respiratory states. This stimulation is correlated qualitatively with elevation of the acetyl-CoA/CoA ratio. With pyruvate alone, pyruvate dehydrogenase flux was maximally stimulated, independent of the energetic state. The latter was suppressed by octanoyl carnitine in all respiratory states, but only marginally.

When pyruvate was in low concentration (~50 to 500 μM), carboxylation was now relatively low in the resting state (concentration-dependent on pyruvate), and was abolished by stepwise stimulation of respiration. This was accompanied by increased production of ketone bodies. Octanoyl carnitine now stimulated pyruvate carboxylase, and suppressed pyruvate dehydrogenase manyfold, such that their relative rates were altered up to ~1,000-fold under some conditions. These effects are correlated with changes (in the same direction) of the acetyl-CoA/CoASH, NADH/NAD+, and ATP/ADP ratios. However, with pyruvate alone, decrease in pyruvate carboxylation again is not correlated with decreased [acetyl-CoA] or decreased acetyl-CoA/CoA. Apparently, other factors such as ADP will decrease carboxylase flux, even in fact of constant or increasing [acetyl-CoA]. It is concluded that the energetic state of the liver cell, and especially the availability of fatty acids, can trigger a very effective coordinated switch in gating pyruvate carbon to oxaloacetate or acetyl-CoA. Endocrine signals may initiate this gating by altering these parameters.

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It is well documented that the metabolic fate of glycolytically derived pyruvate in liver (e.g., Refs. 1–3) and other tissues (4–7) is markedly dependent on dietary or endocrine influences. The primary option for pyruvate metabolism in tissues capable of fat and/or glucose synthesis is at the level of decarboxylation (pyruvate dehydrogenase) and carboxylation (pyruvate carboxylase). A secondary option in liver is the extent to which acetyl-CoA formed in the first reaction is converted to citrate or to ketone bodies. Finally, a third option emerges in the disposition of citrate: it can be oxidized in the citrate cycle, or can be cleaved by citrate lyase (EC 4.1.3.8) with the formation of potential precursors of fatty acids.

The present study was initiated with the intent of devising steady state metabolizing cell-free systems which would be developed systematically to evaluate physiological controls of these central branch points of carbohydrate metabolism. It is known that starvation, diabetes, or fatty acids strongly suppresses pyruvate oxidation by perfused hearts (e.g., 5, 6) and that, in most instances, physiological conditions giving rise to an elevated level of free fatty acids leads to an accelerated rate of gluconeogenesis by liver from substrates giving rise to pyruvate (2, 3). It is apparent that the latter effect is related to an acceleration of carboxylation of pyruvate, coincident with suppression of its decarboxylation.

Studies originating in many laboratories have revealed that pyruvate dehydrogenase activity can be shown to be under control by covalent modification (8, 9) as well as by feedback regulation directly or indirectly by products of the reaction (e.g., NADH, acetyl-CoA, ATP, or the ratios of NADH*/NAD+, acetyl-CoA/CoASH and ATP/ADP, see e.g., Ref. 7 for review). On the other hand, regulation of pyruvate carboxylase might be expected to be under control by ligands or other effectors in a manner which is reciprocal to that for pyruvate dehydrogenase; that is, when pyruvate dehydrogenase activity is stimulated, the activity of pyruvate carboxylase would be suppressed, and vice versa. The principal candidates for regulation of the latter which have been considered are acetyl-CoA, the ATP/ADP ratio, and the concentration of pyruvate (10–13).

Many of the studies on regulation of pyruvate dehydrogenase activity have not involved flux rates directly, but have exposed perfused organs (14–18) or isolated mitochondria (19–24) to varying concentrations of putative effectors, after which the activation state of the enzyme is "fixed" by centrifugation in the cold, or the tissue is rapidly frozen. The activity is then measured under Vmax conditions. Hence, presumably, these studies would reveal alterations in the activity of the enzyme resulting from covalent modification, but would not detect...
alteration of its activity by feedback effects of ligands which may have been present in the tissue. Hansford has recognized this limitation in a recent study of flux through pyruvate dehydrogenase with heart mitochondria (25).

Our strategy has been to measure actual flux rates of these pathways simultaneously under steady state energetic conditions, experimentally designed to mimic the span of respiring and energetic states believed to occur in vivo. We have previously discussed the merits of carrying out in vitro studies with mitochondria between states 4 and 3 (no ADP, and excess ADP present, respectively, Ref. 26). The present study was directed principally to evaluating the fine control of the relative fluxes through pyruvate carboxylase and pyruvate dehydrogenase under the above conditions.

**Materials and Methods**

**Incubations with High Pyruvate Concentration.—** Incubations were carried out for 30 min in media containing (mM) KCl, 90; Tris-HCl, 53; KHCO₃, 16.5; potassium phosphate, 2; ATP, 2; pyruvate, 10; malate, 0.1; acetate, 0.25; DL-3-hydroxybutyrate, 0.5; MgCl₂, 2; and 3 mg/ml of mitochondrial protein at 30 °C under an atmosphere of 5% CO₂, 75% N₂, and 20% O₂. After previous equilibration with this gas mixture at 30 °C, the incubation pH was 7.4. Reactions were started by simultaneous addition of mitochrodria and ATPase. Duplicate 1-ml aliquots from each incubation were taken at 0, 15, and 30 min and transferred to tubes containing 0.3 ml of 20% perchloric acid and placed on ice. After removal of denatured protein and neutralization with KOH, assays for metabolites were carried out. ADP, ATP, and pyruvate were measured within 2 h after termination of incubations. The rates of formation of products did not deviate significantly from linearity during the two time intervals. In control experiments it was found that the 3-hydroxybutyrate/acetate ratio became constant within 5 min and the extramitochondrial ATP/ADP ratio became constant within seconds after the incubations began.

**Incubations with Low Steady State Pyruvate Concentrations.—** It was desirable to carry out experiments with pyruvate in constant concentration and in the physiological range. We therefore sought a pyruvate-generating system linked to lactate dehydrogenase which could remove NADH formed with the latter enzyme, thereby retaining a steady state concentration of pyruvate. A very nearly ideal system was found in the sorbitol-fructose couple (sorbitol dehydrogenase, EC 1.1.1.14). In order to attain constant concentrations of pyruvate, there must be relatively large amounts of both fructose and sorbitol, so that the relative amounts of these substrates, and the absolute amount of lactate, is not significantly altered during the incubation. If we consider reactions 1 and 2,

\[
K_m \text{(as written, pH 7.0)}
\]

(1) \text{Lactate + NAD}^+ \rightleftharpoons \text{pyruvate + NADH}

(2) \text{Fructose + NADH} \rightleftharpoons \text{sorbitol + NAD}^+

it is seen that a relatively high concentration of lactate is necessary to generate the desired concentrations of pyruvate, whereas the \(K_m\) for the sorbitol dehydrogenase reaction is displaced enough from unity to generate higher concentrations of pyruvate than with lactate dehydrogenase alone. The incubation media in this series of experiments was the same, as nearly as possible, as those using a high pyruvrate concentration except that the total osmolality was increased approximately 40 mosm in all conditions. Fructose, sorbitol, and lactate were present in varying concentrations and KC1 was correspondingly reduced. NAD⁺ was 1 mM, and lactate dehydrogenase and sorbitol dehydrogenase were added to 20 and 0.3 I.U./ml, respectively. To generate approximately 0.1 mM pyruvate, the concentrations of fructose, lactate, and sorbitol were 25, 25, and 6 mM, respectively. Higher pyruvate concentrations were attained by increasing the concentrations of lactate and/or fructose. The incubation mixtures were preincubated at 30 °C for 30 min to establish equilibrium of the two-enzyme reaction, then mitochondria were added to start the experiment. Owing to the low specific activity of the sorbitol dehydrogenase, and the high \(K_m\) for its substrates, it was not experimentally practical to maintain true equilibrium after mitochondria were added, and pyruvate was being removed. Thus, the pyruvate concentration was lower than its equilibrium concentration (but essentially constant). Fig. 1 shows a typical curve for pyruvate concentration before and after addition of mitochondria.

**Estimation of Flux Rates.—** Pyruvate carboxylase flux rates were equated with the sum of citrate, malate, and fumarate formed. These intermediates have been shown to constitute more than 90% of all cycle intermediates (27), and are therefore a valid estimation of pyruvate carboxylase flux, since there is negligible production of phosphoenolpyruvate by rat liver mitochondria. When pyruvate was in high initial concentration, pyruvate dehydrogenase flux was readily determined by the expression, pyruvate dehydrogenase flux = \(-\Delta\)pyruvate - \((\Delta\text{citrate} + \Delta\text{malate} + \Delta\text{fumarate})\).

When pyruvate was generated enzymatically it was not possible to use the same calculation for pyruvate dehydrogenase flux as in the experiments with 10 mM pyruvate, so that [1-¹⁴C]lactate was used (0.01 to 0.05 μCi/μmol of lactate). After the incubation media were equilibrated with the gas phase, incubations were carried out in closed vessels for periods of 0, 7.5, and 15 min, and terminated by injection of perchloric acid. [¹⁴C]O₂ was collected in a standard scintillation mixture with appropriate controls for counting efficiency. As has been pointed out previously, (29) radioactivity in [¹⁴C]O₂ liberated from [¹³C]pyruvate is an approximate measure of flux through pyruvate dehydrogenase, but is subject to some errors if a tissue capable of significant carboxylation of pyruvate is studied. That is, after carboxylation of radioactive pyruvate to oxaloacetate, radioactive CO₂ can be subsequently liberated in the citrate cycle, thereby overestimating flux through pyruvate dehydrogenase. We therefore corrected for this error in the following manner: our standard incubation mixture contained 0.1 mM malate, which is adequate for maximal respiration with pyruvate. For each experimental condition, we ran control incubations also with 0.6 and 3.0 mM malate present. Since the specific radioactivity of malate (formed as a result of pyruvate carboxylation) would be diluted by the added malate, one can readily extrapolate to infinite malate concentration (and therefore infinitely low specific activity) and obtain the true flux through pyruvate dehydrogenase. This is illustrated in Fig. 2, in which extremes of the correction are included: i.e. when pyruvate is sole substrate and pyruvate carboxylase flux is very low, the correction for [¹⁴C]O₂ evolution due to the citrate cycle is negligible; in the other extreme, when octanoyl-(-)-carnitine was present, pyruvate carboxylase activity was high, and citrate cycle flux was maximally stimulated with ATPase, the correction for [¹⁴C]O₂ evolution due to the citrate cycle could be as much as 50 to 70%. Intermediate conditions for this correction are also illustrated.

**Estimation of Metabolites.—** ATP, ADP, citrate, malate, fumarate, and pyruvate were measured within 2 h after termination of incubations.

**Fig. 1. Illustrative experiment for generation of steady state pyruvate concentration.** The standard incubation medium contained, in addition, 25 mM fructose, 25 mM potassium lactate, and 6 mM sorbitol. Osmotic pressure was compensated by lowering the concentration of KCl. At zero time, lactate dehydrogenase and sorbitol dehydrogenase were added (see "Materials and Methods"). Curve 2 also contained 1 mM octanoyl-(-)-carnitine. Mitochondria (3 mg of protein/ml) were added at the arrow. The steady state [pyruvate] thus obtained was altered principally by the combined rates of its removal via pyruvate dehydrogenase and pyruvate carboxylase.
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The abbreviation used is: EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N',N"-tetraacetic acid.

of control experiments, was found to be due to the presence of bicarbonate. With glutamate plus malate as substrate the average ATP/ADP ratio in 10 experiments (ATP present but no ATPase) was 3.9 when bicarbonate was not added, and 2.4 if bicarbonate was present. Correspondingly, with pyruvate (10 mM) plus malate (0.1 mM) as substrates, these ratios became 3.0 and 1.5. Hence, these differences were explained in part by intramitochondrial turnover of ATP as a consequence of pyruvate carboxylase. It is also noteworthy that the extramitochondrial ATP/ADP ratio obtained in “state 4” with a high concentration of pyruvate was lower than when the pyruvate concentration was low. This is also explained by the fact that state 4 respiration is stimulated by intramitochondrial turnover of ATP, and, therefore, cannot be termed state 4. To determine the desired rates of respiration, scaled down incubations were carried out using an oxygen-sensitive electrode. State 4 incubations were first begun, followed by various amounts of purified mitochondrial ATPase (F_{1}). It was possible in this way approximately to attain a desired per cent of state 3 respiration, and an approximation of the desired extramitochondrial ATP/ADP ratio. Since there are so many possible variables in attaining any steady state condition, the exact states from one experiment to another could rarely be attained. Hence, the results of averaged determinations from several typical experiments are reported. Experiments as nearly identical in design as possible were repeated at least four times.

All enzymes and coenzymes were purchased from Sigma Chemical Co., St. Louis, Mo. Lithium acetoacetate was prepared according to the method of Hall (37). [8-14C]ATP and NaH[14C]O_{2} were purchased from New England Nuclear. Octanoyl-(-)-carnitine was prepared according to the method of Bremer (38). (-)-Carnitine was a gift of Otsuka Pharmacological Co., Osaka, Japan. Purified mitochondrial ATPase (F_{1}) was prepared by a modification of (39) as previously described (28).

RESULTS

Experiments with a High Concentration of Pyruvate—

Table I shows data from a series of experiments in which the respiratory and energetic steady states are varied over the resting to fully stimulated (respiratory) states when 10 mM pyruvate was present. Note that, as previously reported (28), respiration was maximally stimulated by ATPase when the extramitochondrial ATP/ADP ratio was about 10 or greater. Even in the absence of fatty acid, carboxylation of pyruvate was very rapid in the unstimulated (no ATPase) state, but was progressively diminished on addition of increasing amounts of ATPase. If octanoyl carnitine was present, carboxylation persisted to a greater extent in any given respira-tion-stimulated state (as indicated by the extramitochondrial ATP/ADP ratio). Pyruvate dehydrogenase was presumably fully activated by pyruvate in all states when fatty acid was not present, since its activity was extremely high and virtually identical in all respiratory states. The rates reported here are essentially the same as the fully activated pyruvate dehydrogenase activity reported by Leiter et al. (40). The flux rates through pyruvate dehydrogenase were suppressed by octanoyl carnitine to essentially the same extent in all energetic states, and this suppression was modest (approximately 30%). Hence, flux through pyruvate dehydrogenase, when pyruvate is in high concentration, is relatively insensitive to control via activation-inactivation or by product inhibition, or by both. We therefore conclude, in agreement with Hansford (21) and Hansford and Cohen (25), that flux studies through pyruvate dehydrogenase when pyruvate is in supraphysiological concentrations are of limited physiological significance.

On the other hand, the rate of pyruvate carboxylation did respond to changes in energetic state (Table I), even at saturating concentrations of pyruvate, and this suppression of flux by lowered energetic state (increased respiration, lowered extramitochondrial ATP/ADP ratios) was largely alleviated if octanoyl carnitine was present. Since isolated pyruvate carboxylase is long known to require acetyl-CoA (or a relatively high acetyl-CoA/CoASH ratio) for activity, it is inhibited by ADP, and a decreased intramitochondrial ATP/ADP ratio

![Figure 2](image_url)

**Figure 2.** Correction for variable overestimation of pyruvate dehydrogenase flux due to citrate cycle oxidations releasing 14CO_{2}. Incubations and measurements of 14CO_{2} were carried out in the standard incubation medium containing lactate dehydrogenase and sorbitol dehydrogenase, with adjustments of the KCl concentrations for fructose, sorbitol, and lactate. The concentrations of the latter were 75, 6, and 25 mM, respectively. (Please see “Materials and Methods” for other details.) Points represent the means of 15- and 30-min incubation times, which were linearly related. The concentration range of pyruvate in the four conditions was 0.16 to 0.24 mM. With the high external ATP/ADP ratios, no ATPase was added. In the other two incubations, ATPase was added to obtain the acutely lowered ATP/ADP_{m} ratios as indicated. The range of steady state pyruvate concentrations results from varying total rates of pyruvate removal in the various conditions (pyruvate carboxylase plus pyruvate dehydrogenase fluxes). Open symbols, pyruvate-generating system only; closed symbols, 1 mM octanoyl-(-)-carnitine also present.

3-hydroxybutyrate, acetocacetate, and pyruvate were measured enzymatically by standard procedures as referenced previously (30, 31). Acetyl-CoA and CoASH were measured by the recycling method as described by Allred and Guy (32).

**Preparation of Mitochondria—** Mitochondria were prepared from the livers of chow-fed 200 to 250-g male Wistar rats as described by Johnson and Lardy (33), except that the isolation medium contained 1 mM EGTA. Respiratory controls in media not containing bicarbonate with glutamate plus malate, or 10 mM pyruvate plus 0.1 mM malate, were 6 to 10. However, with the latter substrate, respiratory control ratios of only 2 to 2.5 were attained in the bicarbonate containing medium, resulting principally from the rapid intramitochondrial turnover of ATP as a consequence of carboxylation of pyruvate.

**Intramitochondrial Adenine Nucleotides—** The method for rapid separation of mitochondria and subsequent separation and estimation of nucleotides was exactly the same as previously described (28), with 0.5 mM ATP. The yield of total adenine nucleotides in the pellet after centrifugation through silicone oil in all experiments reported was within the range between 11 to 14 nmole/mg of protein. The ATP/ADP ratios now obtained in ADP-controlled states were consistently less than 2.0, whereas in our (28, 34) and other (35, 36) previous reports, this ratio was approximately 4.0. This difference, in a series
has been correlated with decreased flux through pyruvate carboxylase (11, 12, 41), we have carried out a series of studies similar to those reported in Table I examining these parameters. It was found that when pyruvate alone was substrate, the bulk intramitochondrial ATP/ADP ratio (1.51 ± 0.15 in the resting state) was not significantly altered (p > 0.2, n = 8) if the external ATP/ADP ratio exceeded 10 (fully stimulated respiration). Only when the external ratio was lower (5.5 to 7.0) was the internal ATP/ADP ratio significantly lower (7.73 ± 0.12, pooled data, p < 0.1, n = 6). Similar results were obtained if octanoyl carnitine was also present (data not shown).

Flux ratios through pyruvate carboxylase and pyruvate dehydrogenase (Table I) were only marginally correlated with the acetyl-CoA/CoASH ratio. In four ranges of energetic states with pyruvate as sole substrate (indicated by extramitochondrial ATP/ADP ratios between 35 and 5) the mitochondrial acetyl-CoA/CoASH ratio (1.25 ± 0.13 in the resting state, n = 8) tended to increase as respiration was depressed, although this increase was not statistically significant (with external ATP/ADP ratios over the large range of 15 to 5, the pooled data, n = 12, gave acetyl-CoA/CoASH ratios of 1.47 ± 0.11). Correspondingly, the bulk acetyl-CoA concentration (1.08 ± 0.07 mmole/mg of protein, n = 6) in the unstimulated state (no ATPase) was statistically unaltered in the same range of ATPase-stimulated states (1.19 ± 0.08, n = 8). Octanoyl carnitine elevated the acetyl-CoA/CoASH ratio (to 2.6 ± 0.3, n = 8, p < 0.01).

Thus, these data showed that flux rates through pyruvate dehydrogenase were essentially maximal, and insensitive to changes in energetic or respiratory state, if pyruvate alone was present in saturating concentration. Also, pyruvate dehydrogenase flux was inhibited by octanoyl carnitine only to about 65 to 70% of its maximal rate, and this inhibition was independent of the energetic state of the system.

Although pyruvate carboxylase activity was diminished with increasing respiration rates (and lowered external ATP/ADP ratios), this effect could generally not be correlated with significant lowering of the bulk mitochondrial ATP/ADP, the acetyl-CoA/CoASH ratio, or with the concentration of acetyl-CoASH. It is therefore apparent from these data, briefly presented, that physiological controls of the relative rates of flux at the primary branch point of pyruvate metabolism are, to a large extent, not expressed when pyruvate is in saturating concentration. The data presented serve principally as a point of reference, and contrast to the dramatic effects of energetic state and of competing substrate observed when pyruvate is present in low, steady state concentrations.

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| Other additions | ATP/ADP (external) | ATP/ADP (external) | Flux through PDC (bottom three curves) | Flux through PDC (bottom three curves) |
|-----------------|-------------------|-------------------|--------------------------------------|--------------------------------------|
| None            | 35-41             | 48-43             | 21 ± 2                               | 31 ± 3                               |
|                 | 17-22             | 55-58             | 18 ± 2                               | 32 ± 4                               |
|                 | 12-15             | 72-79             | 13 ± 3                               | 32 ± 5                               |
|                 | 5-7               | 75-80             | 8 ± 2                                | 35 ± 4                               |
| Octanoyl-(−)-carnitine (1 mM) | 36-41 | 48-56 | 38 ± 4 | 24 ± 2 |
|                 | 17-19             | 88-94             | 24 ± 3                               | 21 ± 4                               |
|                 | 13-15             | 89-95             | 26 ± 3                               | 23 ± 3                               |

a Abbreviations: CBX, pyruvate carboxylase (EC 6.4.1.1); PDH, pyruvate dehydrogenase (EC 1.2.4.1).

b Significantly different from control containing no ATPase, p < 0.01.

c Significantly different from corresponding incubations with pyruvate (plus malate) as sole substrate, p < 0.01.

d Significant correlation between increasing rates of ketogenesis from pyruvate and (a) decreased rates of carboxylation of pyruvate (Fig. 3B), and (b) a gradual tendency toward increasing acetyl-CoA/CoASH ratios and absolute bulk concentration of acetyl-CoA (Fig. 3, E and F), suggesting that oxaloacetate may become limiting.

**Fig. 3 summarizes the principal measured parameters when the steady state pyruvate concentrations were varied over three ranges (approximately 0.05, 0.2, and 0.5 mM), with the effects of energetic and respiratory steady state and of octanoyl-(−)-carnitine. It is seen that, even at the lowest pyruvate concentrations, pyruvate dehydrogenase flux was rapid (but not maximal) in the resting state, and was stimulated with increasing respiration and lowered external ATP/ADP ratios. The pattern was similar with higher steady state pyruvate concentrations, although the absolute flux rates were somewhat elevated (Fig. 3A, top three curves). The effect of octanoyl carnitine (bottom three curves) on flux through pyruvate dehydrogenase was now very extensive, especially at the low end of pyruvate concentration. Thus, the rates of flux were reduced as much as 95%, and this inhibition persisted when the energetic states were altered with increasing amounts of ATPase.
FIG. 3. Correlation of the rates of flux through pyruvate dehydrogenase, pyruvate carboxylase, and ketogenesis, and of the mitochondrial oxidation-reduction state, the acetyl-CoA/CoASH ratio, and of the acetyl-CoA concentration with steady state pyruvate concentration, the energetic, or respiratory state (expressed as the extramitochondrial ATP/ADP ratio) and with the presence and absence of a competing fatty acid substrate. Incubations (A through D) were carried out at 30 °C for 0, 7.5, and 15 min in the standard incubation medium, with necessary adjustments for isotonicity required by the presence of varying concentrations (please see "Materials and Methods") of lactate, fructose, and sorbitol needed for the pyruvate-regenerating system. The experiments reported in A through D are taken from a set of four separate experiments. The flux rates taken for the 0- to 7.5- and 0- to 15-min intervals were essentially linear in each case, so that these rates were averaged for determination of fluxes in an individual experiment. The experiments in E and F were from a separate series of four experiments designed to simulate as closely as possible those in A to D. Incubation in E and F were for 10 min, after which they were terminated in HClO₄. The horizontal bars in A and E indicate the range of steady state ATP/ADP ratios in each pooled set. The vertical bars represent the S.E. The closed symbols indicate that only pyruvate (plus 0.1 mM malate) were present as substrate. For open symbols, 1 mM octanoyl-(−)-carnitine was also present. The ranges of steady state pyruvate concentrations were: triangles, 0.05 to 0.08 mM; squares, 0.16 to 0.24 mM; and circles, 0.48 to 0.76 mM. The reason that these values are ranges rather than the same value in each incubation is the result of varying total rates (pyruvate dehydrogenase plus pyruvate carboxylase fluxes) of removal of pyruvate from the generating system (please see Fig. 2). In a few cases, the range of ATP/ADP ratios, and of flux rates, is contained in the symbol for that variable, as indicated in the figures. As mentioned earlier, and in Table I, respiration was maximally stimulated when the extramitochondrial ATP/ADP ratio was about 10 to 15.

For recycling of CoASH. When octanoyl carnitine was also present, ketogenesis was extremely high, and only marginally affected over the range of energetic conditions studied. This high rate of ketogenesis coincided with elevated acetyl-CoA/CoASH and β-hydroxybutyrate/acetoacetate ratios, and relatively high rates of carboxylation of pyruvate (Fig. 3, B to E).

As expected, the mitochondrial NADH/NAD⁺ ratio, as indicated by the observed β-hydroxybutyrate/acetoacetate ratio, was diminished on stimulation of respiration (Fig. 3D). The same was true if octanoyl carnitine was present, but these ratios were substantially higher in all energetic states than with pyruvate as sole substrate.

It is noteworthy that, superficially, pyruvate carboxylase flux is correlated with the acetyl-CoA/CoASH ratio in incubations containing both pyruvate and octanoyl carnitine (Fig. 3, B and E). That is, at any given concentration of pyruvate, carboxylase flux decreases with decreasing acetyl-coA/CoASH ratios. However, there was a trend for elevated acetyl-CoA/CoASH ratios with lower pyruvate concentrations, simultaneous with lower rates of carboxylase flux. The bulk acetyl-CoA concentration was not significantly altered in any of these states. When pyruvate was sole substrate, decreases in the rate of pyruvate carboxylase flux was in no case positively correlated with decreases in the acetyl-CoA/CoASH ratio, or with [acetyl-CoA] (Fig. 3, B, E, and F).

Table II summarizes data on the measured ATP/ADP ratios in mitochondria incubated under conditions similar to
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Table II

| Other additions                  | ATP/ADP (out) | ATP/ADP (in) |
|---------------------------------|---------------|--------------|
| None                            | 34-40         | 1.23 ± 0.06  |
| Octanoyl-(-)-carnitine (1 mM)   | 33-40         | 1.57 ± 0.08  |
|                                 | 14-17         | 1.47 ± 0.12  |
|                                 | 5.4-7.5       | 1.28 ± 0.07  |

* Different from top line in a series, p < 0.01.
* Different from corresponding values without octanoyl carnitine in approximately corresponding energetic state, p < 0.01.

those presented in Fig. 3. As seen in this table, when low concentration of pyruvate was the sole substrate, lowering the external ATP/ADP ratio to that required to stimulate respiration fully (or lower) resulted in significant lowering of the mitochondrial ATP/ADP ratio. If octanoyl-(-)-carnitine was also present, the mitochondrial ATP/ADP ratio was resistant to significant change until the amount of ATPase added exceeded that required for maximum phosphorylating respiration. These data with pyruvate alone are the ranges obtained in individual experiments. Data are from four determinations ± S.E.

DISCUSSION

Since pyruvate is at a central cross-road of metabolism, the control of direction of pyruvate carbon toward oxidation to CO₂ or ketone bodies, or to precursors of new glucose or fatty acid synthesis continues to be a subject of extreme importance, not only for hepatic metabolism, but for whole body homeostasis. We have addressed this important question in the present work. In principal, our experimental design differs from most other studies in that we have studied actual flux rates through pyruvate dehydrogenase, pyruvate carboxylase, and the enzymes of ketogenesis simultaneously, and under respiratory and energetic states designed to mimic the span of those states which are likely to prevail under in vitro conditions. For the first time, it has been possible to provide a strictly constant concentration of pyruvate during an incubation period. By comparing fluxes at the cross-road of pyruvate dehydrogenase and pyruvate carboxylase in the presence of saturating and of physiological concentrations of pyruvate, it should be possible to decide which perturbations are likely to be meaningful under physiological conditions. Since the relative availability of free fatty acids appears to be intrinsic to stimulation of gluconeogenesis, and to inhibition of pyruvate dehydrogenase, we have evaluated the effects of octanoyl-(-)-carnitine on these processes. We have not used long chain fatty acids in order to avoid possible effects of the latter on the adenine nucleotide translocase (43, 44), and therefore possible perturbation of the intramitochondrial ATP/ADP ratio.

In the experiments using saturating pyruvate concentrations, briefly reported, it is evident that any rate limitation of effectors on pyruvate dehydrogenase are reversed by the presence of saturating pyruvate concentrations. The presence of octanoyl-(-)-carnitine suppressed pyruvate dehydrogenase flux to essentially the same extent in all energetic states studied, but this inhibition was not striking. The simplest explanation for this relatively small inhibition is competition for NAD⁺ being recycled by electron transfer. The situation was somewhat different for pyruvate carboxylase flux. Pyruvate alone, at high concentrations, was rapidly carboxylated, and this was suppressed with lowered energetic states. This suppression of pyruvate carboxylation could not, however, be correlated with altered intramitochondrial ATP/ADP or to acetyl-CoA/CoASH ratios. It is possible that changes in the activities of these effectors are underestimated, as briefly discussed below. Rajimand and Bartulis (45) have recently reported a study of the relative rates of carbamoyl phosphate synthesis and pyruvate carboxylase activity (the latter with saturating pyruvate concentration). They noted that both of these ATP-requiring activities were suppressed, corresponding to increased turnover of ADP (hexokinase-glucose) and correspondingly lowered mitochondrial ATP/ADP ratios. Bryla and Niedźwiecka (46) also reported similar results. These data are not directly comparable to those which we report, since these workers have compared extremes of energy state, i.e. with excess ATP, and with excess ADP. Certainly, in the latter case the intramitochondrial ATP/ADP ratio is lowered (28).

The experiments with low steady state pyruvate concentrations are very impressive, with respect to control of the relative fluxes through pyruvate carboxylase and pyruvate dehydrogenase. In all cases, flux through these two pathways was roughly altered in opposite directions by altering the energetic steady state, or by the presence of a competing fatty acid substrate. The relative rates of these two options for pyruvate metabolism, when present as sole substrate, could be shown to be altered by 2 orders of magnitude or greater within the experimental extremes of energetic state. Correspondingly, the presence of octanoyl-(-)-carnitine resulted in equally impressive shifts in the relative rates of carboxylation and decarboxylation of pyruvate. The results are therefore consistent with the idea that the activities of the two enzymes are under physiological regulation by the same effectors, but in opposing directions. Many in vitro studies of the isolated enzymes, taken together, indeed suggest this conclusion.

In the experiments with low concentrations of pyruvate as sole substrate, flux rates through pyruvate carboxylase and pyruvate dehydrogenase do correlate with changes in the mitochondrial ATP/ADP and NADH/NAD⁺ ratios, but these latter changes are certainly not large compared to the observed changes in flux rates. But the results are anomalous in another sense: decreased rates of carboxylation of pyruvate coincided in all instances with no significant change or increases in the concentration of acetyl-CoA and of the acetyl-CoA/CoASH ratio. Thus, under these conditions of low pyruvate concentration, factors other than acetyl-CoA apparently override effects of the latter on pyruvate carboxylation.

The powerful effects of octanoyl-(-)-carnitine on the respective rates of carbon flux through pyruvate carboxylase, pyruvate dehydrogenase, and ketogenesis correlate with in-
creases in the mitochondrial NADH/NAD⁺ acetyl-CoA/CoASH, and ATP/ADP ratios, all of which are putative effectors of pyruvate carboxylation and of pyruvate dehydrogenase. Stimulation of ketogenesis by octanoyl carnitine was not unexpected, owing the known correlation between ketogenesis, the acetyl-CoA/CoASH ratio and the mitochondrial NADH/NAD⁺ ratio (47, 48). In like manner, the increasing production of ketone bodies from pyruvate as sole substrate with lowered energetic state is correlated with increasing acetyl-CoA/CoASH ratios and decreased accumulation of citrate cycle intermediates. As pointed out by others (e.g. Ref. 48), the equilibrium concentration of acetoacetyl-CoA formed in the thiolase reaction is a function of [acetyl-CoA]², so that relatively small changes in the concentration of the latter could have a marked effect on the overall rate of ketogenesis.

Transport of pyruvate into mitochondria has been implicated as a possible rate-determining step for gluconeogenesis and lipogenesis (e.g. Ref. 49). This would suggest that, in some instances, pyruvate carboxylation activity may be limited by the steady state level of intramitochondrial pyruvate. The data in Fig. 3B show that, with pyruvate as sole substrate, pyruvate carboxylation in most energetic states was increased somewhat by increasing the pyruvate concentration over the range between approximately 0.05 and 0.5 mM. Pyruvate dehydrogenase activity was likewise stimulated in the range of pyruvate concentrations between about 0.05 and 0.20 mM, suggesting perhaps that pyruvate transport, or simply its intramitochondrial concentration may be limiting for both of these activities when pyruvate is low physiological concentration. The fact that ketone bodies tend to accumulate as respiration is stimulated, even at the lowest pyruvate concentration, would tend to rule out an important concentration dependence for pyruvate dehydrogenase activity. This argument, however, is not valid directly for considerations of concentration limitation for pyruvate carboxylation activity. Under our incubation conditions of controlled respiration in presence of octanoyl carnitine, pyruvate carboxylation activity was extremely high, and was relatively insensitive to pyruvate concentrations. In fact, the flux rates reported here are calculated to be approximately equal to the total activity reported for pyruvate carboxylation in rat liver (50). The Kₘ of the pyruvate carrier for pyruvate has been estimated to be in the range 0.2 mM or higher (c.f. Ref. 7) and has been shown to catalyze uptake of pyruvate in exchange for acetoacetate (51). Thus, the intriguing possibility remains that one important mode of action of fatty acids on hepatic pyruvate metabolism is to stimulate pyruvate carboxylation through accelerated transport of pyruvate during ketogenesis. An additional possibility for an effect on pyruvate carboxylation by octanoyl carnitine oxidation which has not been considered is an effect of acyl-CoA intermediates. This is very unlikely, however, since the sums of acetyl-CoA plus CoASH in all of the experiments shown in Fig. 3 were statistically identical (data not shown), making it extremely unlikely that significant acyl-CoA intermediates of octanoyl carnitine accumulate.

Interpretation of the correlation of measured variables with the intramitochondrial ATP/ADP ratio is, of course, dependent on the validity of the method used for these determinations. In previous reports (e.g. 28) and the present study, with saturating concentration of reducing substrates, the measured intramitochondrial ATP/ADP ratio was resistant to significant changes until the extramitochondrial ATP/ADP ratio is reduced to values lower than required for maximal stimulation of respiration, whereas with limiting pyruvate (plus catalytic concentration of malate) as sole substrate, the mitochondrial ATP/ADP ratio was lowered significantly with increased respiration in what is believed to be “physiological” energetic states. Although the silicone oil separation remains the most widely used and documented (28, 52) rapid procedure for separation of mitochondria from an incubating medium, several workers have questioned its validity (e.g. 21, 42, 53). Hansford (21) has logically suggested that, if the time interval before quenching were long enough, the ratio of mitochondria to external ATPase (or hexokinase) would be increased during the separation period, resulting in a reversion toward resting respiration. Although this is a crucial point which is well taken, we believe that control experiments published earlier (28) at least qualitatively show that this scepticism is unwarranted: first, the intramitochondrial ATP/ADP did respond to more extreme (unphysiological) variations in the external ratio; and most compellingly, (b) ATPase-stimulated respiratory states were correlated with extramitochondrial ATP/ADP ratios and intramitochondrial NADH/NAD⁺ ratios, the latter being increased with increasing rates of respiration. Whether the mitochondria were quenched immediately in acid, or were centrifuged through silicone, the measured mitochondrial [NAD⁺] was approximately the same in both conditions (28). Brawand et al. (42) have suggested another possible error in the determinations. They have suggested that the correction for nucleotides in the extra-matrix water carried down with the mitochondria is large enough to make the method insensitive to changes in the nucleotides in the mitochondrial matrix space. In previous work (28) the corrected matrix ATP/ADP ratio was statistically the same, whether the extramitochondrial adenine nucleotide concentration was 0.1, 0.5, or 2.0 mM. In the present experiments, 0.5 mM adenine nucleotides were added to the incubation medium for determination of internal nucleotides. This amounts to about a 10 to 15% correction for nucleotides in the extramatrix water. Finally, since this work was completed Wanders et al. (53) have also suggested, following Hansford (21), that there is a time-dependent blunting of changes in the mitochondrial ATP/ADP during separation, especially when the phosphorylation rate is high. They have used highly unphysiological conditions to demonstrate this by incubating mitochondria with very low concentrations of adenine nucleotides plus glucose and high activities of hexokinase, such that [ATP] in the suspending medium was virtually zero, and as a result the matrix ATP/ADP ratio also approached zero. Although the matrix ATP/ADP ratio was shown to change together with malonate-inhibited electron transfer, the measured matrix ATP/ADP ratios tended to be higher when measured after silicone separation than after immediate quenching with acid. Thus, even under these very extreme conditions, the silicone separation method detected changes in intramitochondrial nucleotides, although the changes were somewhat dampened under these special conditions.

The most important conclusions to be drawn from the present work are that, when pyruvate is in the physiological or near-physiological range of concentrations, the flux rates through pyruvate carboxylation and pyruvate dehydrogenase are very strongly influenced in a reciprocal manner by altering the respiring and energetic steady states through the range which is probably seen under physiological conditions. Even more extensive is the effect of competing fatty acid substrate on stimulation of pyruvate carboxylation, and suppression of pyruvate dehydrogenase flux, such that the relative rates of these two reactions are altered by 2 to 3 orders of magnitude. The measured concentrations or ratios of putative effectors of these reactions change generally, but not always, in the same direction as expected if they were affecting flux rates. When pyruvate was the sole substrate, however, pyruvate carbox-
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pylase flux was not correlated with acetyl-CoA or the acetyl-CoA/CoASH ratio but, in agreement with Brawand et al. (42), was apparently suppressed by lowered mitochondrial ATP/ADP ratio. As in Ref. 42, these changes were not large. As discussed above, it remains a possibility that changes in mitochondrial nucleotides may be blunted during the quenching procedure, but this effect is apparently slight at most. An additional possibility which cannot be ruled out at present is that part of the adenine nucleotides is bound (see e.g. Refs. 54 and 55), and is therefore not accessible as effector(s) of the enzyme fluxes being studied here. Similarly, evidence has been presented suggesting that acetyl-CoA and CoASH are not uniformly distributed in the mitochondrial matrix (13). If this is the case, the magnitude of bulk changes in effector concentrations may not reflect true changes in their metabolically active forms.

It is further emphasized that the exquisite control of the disposition of pyruvate carbon is observed only when pyruvate is in relatively low steady state concentrations.

REFERENCES

1. Krebs, H. A. (1964) Proc. R. Soc. Lond. B. Biol. Sci. 159, 545-564
2. Struck, E., Ashmore, J., and Wieland, O. (1965) Biochem. Z. 343,107-110
3. Garcia, A., Williamson, J. R., and Cahill, G. F., Jr. (1966) Diabetes 15, 188-193
4. Williamson, J. R., and Krebs, H. A. (1961) Biochem. J. 80, 540-547
5. Garland, P. B., Newsholme, E. A., and Randle, P. J. (1964) Biochem. J. 93, 665-678
6. Davis, E. J., and Quastel, J. H. (1964) Can. J. Biochem. 42, 1605-1621
7. Denton, R. M., Randle, P. J., Bridges, G. J., Cooper, R. H., Kerley, A. L., Pask, H. T., Severson, D. L., Stansbie, D., and Whitehouse, S. (1975) Mol. Cell. Biochem. 9, 27-53
8. Lim, T. C., Pettit, F. H., and Reed, L. J. (1969) Proc. Natl. Acad. Sci. U. S. A. 62, 234-241
9. Wieland, O. H., and Siess, E. A. (1970) Proc. Natl. Acad. Sci. U. S. A. 65, 947-954
10. Utter, M. F., and Kech, D. B. (1963) J. Biol. Chem. 238, 2603-2608
11. Walter, P., Paetkau, V., and Lardy, H. A. (1966) J. Biol. Chem. 241, 2523-2532
12. Stucki, J. W., Brawand, F., and Walter, P. (1972) Eur. J. Biochem. 27, 181-191
13. Barratt, G. J., Zander, G. L., and Utter, M. F. (1976) in Gluconeogenesis: Its Regulation in Mammalian Species (Hanson, R. W., and Mehlm, M. A., eds) pp. 3-46, John Wiley and Sons, New York
14. Wieland, O., Funcke, H. V., and Löffler, G. (1971) FEBS Lett. 15, 295-298
15. Wieland, O., Paetkau, V., and Löffler, G. (1972) Eur. J. Biochem. 26, 426-433
16. Olson, M. S., Dennis, S. C., DeBuysere, M. S., and Padma, A. (1978) J. Biol. Chem. 253, 7399-7375
17. McAllister, R., Allison, S. P., and Randle, P. J. (1973) Biochem. J. 134, 1067-1081
18. Kerney, A. L., Randle, P. J., Cooper, R. H., Whitehouse, S., Pask, H. T., and Denton, R. M. (1976) Biochem. J. 154, 327-348
19. Portenheuser, R., and Wieland, O. (1972) Eur. J. Biochem. 31, 308-314
20. Portenheuser, R., and Wieland, O. (1977) Hoppe-Seyler's Z. Physiol. Chem. 358, 647-656
21. Hafre, R. G. (1977) J. Biol. Chem. 252, 1552-1560
22. Batenburg, J. J., and Olson, M. S. (1978) J. Biol. Chem. 251, 1364-1370
23. Schuster, S. M., and Olson, M. S. (1972) J. Biol. Chem. 247, 5088-5094
24. Batenburg, J. J., and Olson, M. S. (1975) Biochem. Biophys. Res. Commun. 66, 533-540
25. Hafre, R. G., and Cohen, L. (1978) Arch. Biochem. Biophys. 191, 65-81
26. Chance, B., and Williams, G. R. (1956) Adv. Enzymol. 17, 65-134
27. Walter, P., and Stucki, J. W. (1970) Eur. J. Biochem. 12, 506-519
28. Davis, E. J., and lumeng, L. (1975) J. Biol. Chem. 250, 2275-2282
29. Dennis, S. C., DeBuysere, M., Scholz, R., and Olson, M. S. (1978) J. Biol. Chem. 253, 2229-2237
30. Lee, S.-H., and Davis, E. J. (1979) J. Biol. Chem. 254, 420-430
31. Davis, E. J., and Gibson, D. M. (1969) J. Biol. Chem. 244, 161-170
32. Allred, J. B., and Guy, D. G. (1969) Anal. Biochem. 29, 293-299
33. Johnson, D., and Lardy, H. A. (1967) Methods Enzymol. 10, 94-96
34. Davis, E. J., Lumeng, L., and Bottoms, D. (1974) FEBS Lett. 39, 9-12
35. Heldt, H. W., Klingenberg, M., and Milovancev, M. (1972) Eur. J. Biochem. 30, 434-440
36. Slater, E. C., Rosing, J., and Mol, A. (1973) Biochim. Biophys. Acta 252, 534-553
37. Hall, L. M. (1962) Anal. Biochem. 3, 75-80
38. Bremer, J. (1968) Biochem. Prep. 12, 69-73
39. Penefsky, H. S. (1967) Methods Enzymol. 10, 522-526
40. Lieter, A. B., Weinberg, M., Isoshahi, F., and Utter, M. F. (1978) J. Biol. Chem. 253, 2716-2723
41. Walter, P. (1976) in Gluconeogenesis: Its Regulation in Mammalian Species (Hanson, R. W., and Mehlm, M. A., eds) pp. 239-265, John Wiley and Sons, New York
42. Brawand, F., Folly, G., and Walter, P. (1980) Biochim. Biophys. Acta 590, 285-289
43. Pande, S. V., and Blancaer, M. C. (1971) J. Biol. Chem. 246, 402-411
44. Shug, A., Lerner, E., Elson, Ch., and Shrago, E. (1971) Biochim. Biophys. Res. Commun. 43, 557-563
45. Rajtman, L., and Bartulis, T. (1979) Arch. Biochem. Biophys. 195, 188-197
46. Bryla, J., and Niedzwiecka, A. (1979) Int. J. Biochem. 10, 235-239
47. Garland, P. B., Shepherd, D., and Yates, D. W. (1965) Biochem. J. 97, 587-594
48. Lopes-Cardozo, M., and van den Berg, S. G. (1974) Biochim. Biophys. Acta 357, 53-62
49. Halestrap, A. P., Scott, R. D., and Thomas, A. P. (1980) Int. J. Biochem. 11, 97-105
50. Weinberg, M. B., and Utter, M. F. (1980) Biochem. J. 188, 601-608
51. Halestrap, A. P. (1978) Biochem. J. 172, 377-387
52. Pfaff, E. (1965) Ph.D. thesis, Marburg
53. Wandsers, R. J. A., van Woerkom, G. M., Nooteboom, R. F., Meijer, A. J., and Tager, J. M. (1981) Eur. J. Biochem. 113, 295-302
54. Matlil, M. A., Shannon, W. A., and Sirere, P. A. (1977) Arch. Biochem. Biophys. 178, 396-407
55. Lusty, C. J. (1978) Eur. J. Biochem. 85, 373-383