Regulation of Hepatic Gluconeogenesis in the Guinea Pig by Fatty Acids and Ammonia*

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Octanoate and L-palmitylcarnitine inhibited the synthesis of P-enolpyruvate from α-ketoglutarate and malate by isolated guinea pig liver mitochondria. A 50% reduction in P-enolpyruvate formation was obtained with 0.1 to 0.2 mM octanoate or with 0.06 to 0.10 mM L-palmitylcarnitine. At these concentrations, oxidative phosphorylation remained intact and only much higher concentrations of fatty acids altered this process. The addition of NH₄Cl in the presence of malate and increasing concentrations of α-ketoglutarate (or vice versa) enhanced the formation of glutamate, aspartate, and P-enolpyruvate. The addition of increasing concentrations of NH₄Cl in the presence of fixed amounts of malate and α-ketoglutarate had a similar effect. Furthermore, the inhibition of P-enolpyruvate synthesis by fatty acids and the reduction of the acetoacetate to β-hydroxybutyrate ratio were reversed by the addition of NH₄Cl. Cycloheximide, which blocks energy transfer at site 1 of the respiratory chain, decreased P-enolpyruvate formation. When cycloheximide and either octanoate or L-palmitylcarnitine were added together, there was an even greater reduction in P-enolpyruvate synthesis from either malate or α-ketoglutarate than was noted with either fatty acid alone. Since cycloheximide lowers the rate of ATP synthesis this may in turn reduce P-enolpyruvate formation by a mechanism independent of changes in the mitochondrial NAD⁺/NADH ratio caused by fatty acids.

In the isolated perfused liver metabolizing lactate, the inhibitory effect of octanoate on gluconeogenesis was partially relieved by the addition of 1 mM NH₄Cl, but remained unchanged in the presence of 2 mM NH₄Cl, despite a highly oxidized NAD⁺/NADH ratio in the mitochondria. In contrast to glucose synthesis, urea formation was markedly increased during the infusion of 1 mM as well as 2 mM NH₄Cl. After cessation of NH₄Cl infusion, there was an increase in glucose production, to a rate as high as that observed in the absence of octanoate. This increase was accompanied by the disappearance of alanine, aspartate, and glutamate which had been stored in the liver during NH₄Cl infusion. Urea synthesis also decreased progressively. These results indicate that gluconeogenesis in guinea pig liver is regulated, in part, by alterations in the mitochondrial oxidation-reduction state. However, the modulation of this effect by changing the concentrations of intermediates of the aspartate aminotransferase reaction indicates competition for oxaloacetate between the aminotransferase reaction and P-enolpyruvate carboxykinase.

Species differences in the regulation of hepatic gluconeogenesis have been well characterized (1, 2). A comparison of rat and guinea pig livers has shown that the presence of a mitochondrial P-enolpyruvate carboxykinase in the latter species is partly responsible for a number of differences in hepatic glucose synthesis (3–7). Fatty acid oxidation by guinea pig liver mitochondria shifts the NAD⁺/NADH ratio toward reduction and markedly reduces the rate of P-enolpyruvate formation (3, 4, 8). Furthermore, octanoate infusion into perfused guinea pig livers markedly diminishes gluconeogenesis from lactate and alanine (4, 8), but stimulates this process in rat liver (9–12). The inhibitory effect of fatty acids on gluconeogenesis coincides with a decrease in the mitochondrial NAD⁺/NADH ratio as calculated from the equilibrium of β-hydroxybutyrate dehydrogenase and glutamate dehydrogenase reactions.

While there is no doubt that fatty acids can inhibit hepatic gluconeogenesis in guinea pig liver in vitro, the physiological significance of this finding has not been established. During fasting the oxidation of fatty acids by the liver is enhanced, yet gluconeogenesis increases (4). Furthermore, the mitochondrial NAD⁺/NADH ratio in guinea pig livers, freeze-clamped in vivo, shifts markedly toward oxidation after 48 to 72 hours of fasting (13). The relationship between this shift toward
oxidation in the mitochondrial oxidation-reduction state and the oxidation of fatty acids has not been clearly delineated. Other factors such as the metabolism of amino acids and of ammonia in the liver might also contribute to changes to the in vivo mitochondrial oxidation-reduction state. In this report we have studied the interaction of ammonia metabolism with gluconeogenesis and ureogenesis in perfused guinea pig liver. Our results indicate that ammonia, at concentrations close to those noted in intact liver, will reverse the inhibitory effect of fatty acids on glucose synthesis from lactate by perfused guinea pig liver.

EXPERIMENTAL PROCEDURE

Materials—Lactate dehydrogenase (EC 1.1.1.27), β-hydroxybutyrate dehydrogenase (EC 1.1.1.30), pyruvate kinase (EC 2.7.1.40), glucose-6-phosphate-dehydrogenase (EC 1.1.1.49), glutamate dehydrogenase (EC 1.4.1.12), and adenylate kinase (EC 2.7.4.3) as well as NADH, NADP+, ATP, and ADP were obtained from Boehringer Mannheim Corp. Octanoc acid, L-lactic acid, aspartate, glutamate, cycloheximide, aminoxyacetate, and urease (EC 3.5.1.5) type VI were purchased from Sigma Chemical Co. Malate, α-ketoglutarate, N,N'-dihydroxyethylpiperazine-N'-2-ethanesulfonic acid were from Calbiochem and Fermentozyme 952 DM was from Fermeco Laboratories. L-Palmitylcarnitine was generously supplied by Dr. I. Kizawa of Otsuka Pharmaceuticals, Tokushima, Japan.

Animals—Male guinea pigs (200 to 250 g) of the Hartley strain were fed ad libitum on Wayne guinea-pig chow supplemented with fresh greens daily.

Isolation and Incubation of Mitochondria—Mitochondria from guinea pig were isolated by differential centrifugation in 0.32 m sucrose and 4 mM Hepes buffer at pH 7.35. After separation from nuclei (700 × g for 10 min), the mitochondria were sedimented by centrifugation at 12,000 × g for 20 min, then washed twice with 12 volumes of the same buffer per g of liver used. Mitochondria (approximately 12 mg of protein) were incubated in 3 ml of 160 mM sucrose, 2 mM Hepes, 20 mM potassium phosphate (pH 7.5), 6.6 mM MgCl2, 3.3 mM ADP, and the various substrates as indicated. After 10 min at 30° the incubation was stopped with chilled perchoric acid (final concentration, 6% v/v) and the extracts were neutralized with potassium bicarbonate and centrifuged. The concentrations of the various metabolites in the extracts were determined enzymically. Mitochondrial proteins were measured by their ultraviolet absorption with bovine serum albumin as a standard (14). For studies of oxygen consumption, mitochondria were incubated in a thermostated cuvette with a Clark-type oxygen electrode. Details of this procedure are described in the legend to Fig. 2. The average respiratory control ratio measured in control incubation of mitochondria without fatty acids was about 7.0.

Liver Perfusion—Guinea pigs, fasted for 48 hours were anesthetized with sodium pentobarbital (Nembutal) injected intraperitoneally (50 mg/kg). The liver was perfused using the hemoglobin-free non-recycling system described previously (15-17). During the perfusion, the arteriovenous difference in oxygen consumption was monitored continuously with dual oxygen electrodes. Samples of the perfusate were taken for the determination of glucose, acetocetate, β-hydroxybutyrate, and various amino acids. In some experiments the left lateral lobe of the liver was freeze-clamped to terminate the perfusion, powdered in a mortar cooled with liquid nitrogen, and homogenized with perchloric acid, and the metabolites were measured in neutralized extracts.

Determination of Metabolites—Glucose (18) and amino acids (19) were determined using a Technicon autoanalyzer. Urea was measured by the method of Bernt and Bergmeyer using NH4+-free urease (20). All samples were previously treated with Permutit to remove free ammonia (21). In experiments with mitochondria, enzymic methods were used for the determination of aspartate (21) and glutamate (22). ATP (23), ADP and AMP (24), P-enolpyruvate (25), acetocetate (26), and β-hydroxybutyrate were determined enzymically. This was a modification of the method of Williamson and Mellanby (27) using final concentrations of 7 mM disodium ethylenediaminetetraacetate, 15 mM MgCl2, 150 mM Tris, 500 mM hydrazine hydrate, 1 mM NAD+, and 0.4 unit/ml of 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) at pH 8.6.

RESULTS

The synthesis of P-enolpyruvate from either malate or α-ketoglutarate by isolated guinea pig liver mitochondria is markedly inhibited by the oxidation of octanoate (Fig. 1). A 50% inhibition of P-enolpyruvate formation from malate was noted at 0.2 mM octanoate, whereas the same degree of inhibition with α-ketoglutarate as substrate required only 0.1 mM octanoate. This inhibition was not due to an uncoupling of oxidative phosphorylation since the levels of AMP remained constant over the entire concentration range of octanoate added to the incubation medium. Also, at the lowest concentration of octanoate there was a sharp increase in ATP formation when malate was the substrate. This activation was consistent with our observations of oxygen consumption by guinea pig liver mitochondria metabolizing malate plus glutamate (Fig. 2). The lack of increase in AMP formation confirmed the functional stability of these mitochondria at the concentrations of fatty acid used. We also noted (Fig. 3) a 50% inhibition of P-enolpyruvate formation with approximately 0.08 and 0.06 mM L-palmitylcarnitine in the presence of malate and α-ketoglutarate, respectively. The high rate of ATP synthesis and the absence of changes in the concentration of AMP indicated that the mitochondria remained coupled while metabolizing either fatty acid.

Work in our laboratory (7) and by Bryla (28) has indicated that alterations in the mitochondrial oxidation-reduction state during the oxidation of fatty acids is partly responsible for the marked reduction in the rate of mitochondrial P-enolpyruvate synthesis by isolated guinea pig, human, and rabbit liver mitochondria metabolizing a variety of substrates. To test this hypothesis further, we measured the effect of NH4Cl on P-enolpyruvate formation from α-ketoglutarate and malate (Fig. 4). NH4Cl should cause an oxidation of the mitochondrial oxidation-reduction state by shifting the equilibrium of the glutamate dehydrogenase reaction toward glutamate formation with a resultant net generation of NAD+. Such a shift would be dependent on a source of α-ketoglutarate and should stimulate P-enolpyruvate synthesis. As shown in Fig. 4, NH4Cl in a concentration of up to 2 mM increased P-enolpyruvate forma-

![Fig. 1. The effect of octanoate on P-enolpyruvate ATP and AMP formation by isolated guinea pig liver mitochondria. Mitochondria (10 to 12 mg of protein) were incubated in 3 ml of 160 mM sucrose, 2 mM Hepes, 3.3 mM ADP, 6.6 mM MgCl2, and 20 mM potassium phosphate, pH 7.5, with malate and α-ketoglutarate present at a concentration of 2 mM and octanoate as indicated in the figure. The bars represent the standard error of the mean for five experiments.](http://www.jbc.org/)
FIG. 2. Oxygen consumption by guinea pig liver mitochondria suspensions metabolizing malate and glutamate. Mitochondria (approximately 6 mg) were incubated at 25°C in 2 ml of 0.25 M sucrose, containing 5 mM malate, 5 mM glutamate, 5 mM MgCl₂, 3.2 mM Hepes, and 5 mM potassium phosphate at pH 7.5. ADP (0.25 mM) was added at times shown by the arrow while octanoate and L-palmitylcarnitine were added at concentrations indicated in the figure, at the beginning of the incubation.

FIG. 3. The effect of L-palmitylcarnitine on P-enolpyruvate, ATP, and AMP formation by isolated guinea pig liver mitochondria. Mitochondria were incubated as described in Fig. 1. The bars represent the standard error of the mean for five experiments.

tion from malate plus α-ketoglutarate, so that at the highest concentration of NH₄Cl (2 mM), the rate of P-enolpyruvate synthesis in the presence of 0.2 mM octanoate was equal to the rates of synthesis in the absence of fatty acid. Both glutamate and aspartate formation were stimulated by addition of NH₄Cl, indicating that the glutamate dehydrogenase equilibrium was shifted toward glutamate synthesis and that subsequent transamination of glutamate with oxalacetate via aspartate aminotransferase also led to a buildup of aspartate. The stoichiometry of this effect of NH₄Cl on isolated guinea pig liver mitochondria is shown in Fig. 5. In these experiments 2 mM NH₄Cl was used at a fixed concentration with either malate or α-ketoglutarate present at 2 mM. In each experiment, the substrate in variable concentration was the limiting factor for the production of P-enolpyruvate or glutamate and aspartate. The negative effect of octanoate on P-enolpyruvate formation was also counteracted by the positive effect of NH₄Cl. Optimum rates of P-enolpyruvate formation were noted when α-ketoglutarate was present at a fixed concentration of 2 mM and the levels of malate were increased to 4 mM. Fig. 5 also shows that aspartate aminotransferase can compete effectively with P-enolpyruvate carboxykinase since aspartate synthesis also increased markedly. Malate was a better source of carbon for both P-enolpyruvate and aspartate synthesis than was α-ketoglutarate, probably reflecting a more rapid rate of oxalacetate formation from malate by guinea pig liver mitochondria. Also, the synthesis of glutamate was always higher in the presence of octanoate, indicating that NADH formed by fatty acid oxidation was being used (in the presence of NH₄Cl) to drive the glutamate dehydrogenase reaction toward glutamate synthesis.

Fig. 4. The effect of NH₄Cl on P-enolpyruvate, glutamate, and aspartate formation by isolated guinea pig liver mitochondria. Mitochondria were incubated as described in Fig. 1 except that 2 mM malate and 2 mM α-ketoglutarate were added together as substrates. The formation of P-enolpyruvate (A—A), glutamate, (O—O), and aspartate (■—■) with increasing concentrations of NH₄Cl was determined in the presence and absence of octanoate. The bars represent the standard error of the mean for four experiments.

Fig. 5. The effect of increasing concentrations of α-ketoglutarate and malate on the formation P-enolpyruvate, aspartate (ASP) and glutamate (GLU) by isolated guinea pig liver mitochondria. Mitochondria (10 to 12 mg of protein) were incubated as described in Fig. 1. The incubation medium contained 2 mM NH₄Cl and 2 mM malate with increasing concentrations of α-ketoglutarate or 2 mM NH₄Cl and 2 mM α-ketoglutarate with increasing concentrations of malate as indicated in the figure. The open symbols represent intermediates measured in the presence of 0.2 mM octanoate and closed symbols are controls without octanoate. Aspartate, (■); glutamate, (O); P-enolpyruvate in the absence of NH₄Cl (□) and in the presence of (Δ, ▲) 2 mM NH₄Cl. The bars represent the standard error of the mean for four experiments.
The relationship between the rate of P-enolpyruvate synthesis and the production of ketone bodies, glutamate, and aspartate by guinea pig liver mitochondria metabolizing 2 mM malate plus 2 mM α-ketoglutarate is further demonstrated in Table I. Octanoate and L-palmitylcarnitine both decreased the rate of P-enolpyruvate formation and this effect could be reversed by NH₄Cl. Cycloheximide, which blocks energy transfer at site 1 of the respiratory chain (29), also decreased P-enolpyruvate synthesis, but when added to mitochondria together with fatty acids, there was an even greater decrease in P-enolpyruvate formation. This effect is probably related to the profound reduction in the oxidation-reduction state of the mitochondria when both cycloheximide and either octanoate or L-palmitylcarnitine were present together. The ratio of acetocetate to β-hydroxybutyrate decreased from 3.7 in control incubations to 0.9 when L-palmitylcarnitine and cycloheximide were combined. This was accompanied by a decrease in ATP synthesis in state 3 when cycloheximide and L-palmitylcarnitine were present, again consistent with a block of energy transfer at site 1 of the respiratory chain. The addition of NH₄Cl to the mitochondrial incubation medium partly relieved P-enolpyruvate formation in the presence of cycloheximide without affecting the markedly reduced rate of ATP formation. In those experiments where fatty acids and NH₄Cl were present together, the rate of glutamate, but not aspartate, synthesis could be markedly increased by cycloheximide. Since the level of ATP synthesis remained reduced after cycloheximide addition, it is reasonable to assume that the increased glutamate formation was due to a further increase in NADH generation caused by a block of site 1. P-enolpyruvate synthesis was also decreased, reflecting the reduction in the mitochondrial oxidation state as shown by the decreased acetocetate to β-hydroxybutyrate ratio (2.5 with cycloheximide, NH₄Cl, and octanoate) versus 5.9 with octanoate plus NH₄Cl.

The direct transamination of oxalacetate to aspartate by guinea pig liver mitochondria was markedly inhibited by aminooxyacetic acid, a compound which blocks aspartate aminotransferase (30, 31). When mitochondria were metabolizing malate and α-ketoglutarate, NH₄Cl addition resulted in the formation of 76 nmol of aspartate/mg of mitochondrial protein in 10 min (Table I). Aminooxyacetic acid reduced the rate of aspartate synthesis to 6.1 nmol/mg of protein, whereas the synthesis of glutamate and of P-enolpyruvate was not blocked.

The inhibition of aspartate aminotransferase by aminooxyacetic acid caused an increase in P-enolpyruvate synthesis by guinea pig liver mitochondria when NH₄Cl was included in the incubation medium. Thus the rate of P-enolpyruvate formation increased from 125 nmol/mg of protein in 10 min with NH₄Cl present to 151 nmol when aminooxyacetic acid and NH₄Cl were added together. This probably reflected the decreased use of oxalacetate for aspartate synthesis, thereby diverting substrate for the P-enolpyruvate carboxykinase reaction.

There is a good correlation between the control of P-enolpyruvate synthesis by guinea pig liver mitochondria and the regulation of gluconeogenesis in perfused guinea pig livers (4, 8, 32). Octanoate infusion sharply decreased glucose synthesis from lactate (Fig. 6) while 1 mM NH₄Cl added together with octanoate reversed the pattern of inhibition and caused a return of glucose synthesis to levels noted before octanoate infusion. This effect of NH₄Cl on gluconeogenesis was concentration-dependent since the infusion of 2 mM NH₄Cl together with octanoate did not overcome the inhibition caused by the fatty acid until NH₄Cl infusion was terminated.

### Table I

**Relationship between intramitochondrial oxidation reduction state and P-enolpyruvate formation**

Mitochondria were incubated as described in Fig. 1 except that 2 mM malate and 2 mM α-ketoglutarate were added together to each incubation. NH₄Cl, when present, was added at a concentration of 2 mM, cycloheximide at 5 mM, octanoate at 0.2 mM, and L-palmitylcarnitine at 0.1 mM. Values are the means ± standard error for three experiments.

| Additions                  | P-enolpyruvate (nmol/mg protein/10 min) | Acetocetate | β-Hydroxybutyrate | Acetoacetate to BOHB | ATP | Aspartate | Glutamate |
|----------------------------|----------------------------------------|-------------|------------------|----------------------|-----|-----------|-----------|
| Control                    | 81 ± 2.4                               | 2.4 ± 0.2   | 0.7 ± 0.08       | 3.7                   | 430 ± 16 |           |           |
| Cycloheximide              | 35 ± 1.3                               |             |                  |                       |     |           |           |
| NH₄Cl                      | 125 ± 1.9                              |             |                  |                       |     |           |           |
| Cycloheximide + NH₄Cl      | 71 ± 3.0                               |             |                  |                       |     |           |           |
| Octanoate                  | 5/4 ± 4.0                              | 10.8 ± 1.4  |                  | 4.6 ± 12              |     |           |           |
| + L-Palmitylcarnitine      | 38 ± 3.7                               | 6.0 ± 0.5   |                  | 1.9 ± 0.2             |     |           |           |
| Octanoate + cycloheximide  | 12 ± 1.4                               | 10.0 ± 1.2  |                  | 0.7 ± 0.7             |     |           |           |
| + L-Palmitylcarnitine      | 11 ± 0.7                               | 10.1 ± 1.6  |                  | 0.9 ± 0.7             |     |           |           |
| Octanoate + NH₄Cl          | 76 ± 8.3                               | 8.9 ± 0.8   |                  | 5.9 ± 0.2             |     |           |           |
| + L-Palmitylcarnitine      | 83 ± 4.7                               | 7.5 ± 0.3   |                  | 4.5 ± 0.9             |     |           |           |
| Octanoate + NH₄Cl + cyclo- | 37 ± 6.2                               | 9.7 ± 2.3   |                  | 2.6 ± 0.9             |     |           |           |
| heximide                   | + L-Palmitylcarnitine + cycloheximide   | 35 ± 1.8    | 10.5 ± 1.7       | 2.5 ± 0.7             |     |           |           |
| Aminooxyacetic acid        | 85 ± 1.8                               |             |                  |                       |     |           |           |
| Aminooxyacetic acid + NH₄Cl| 161 ± 13.0                             |             |                  |                       |     |           |           |
| Aminooxyacetic acid + L-   | 39 ± 3.8                               |             |                  |                       |     |           |           |
| Palmitylcarnitine          | 93 ± 8.4                               |             |                  |                       |     |           |           |

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At this time there was a rapid return of gluconeogenesis to normal levels despite the presence of octanoate. NH₄Cl caused a shift in the mitochondrial oxidation-reduction state toward oxidation as reflected by a change in the acetoacetate to β-hydroxybutyrate ratio from 4 before, to approximately 6, after 15 min NH₄Cl infusion. Urea synthesis was sharply increased by NH₄Cl infusion in a manner roughly proportional to the concentration of NH₄Cl. Thus 0.9 μmol of urea/min/g of liver were formed during infusion of 1 mM NH₄Cl while 1.5 μmol were noted with 2 mM NH₄Cl.

In order to clarify the differential effect of 1 and 2 mM NH₄Cl on gluconeogenesis, livers were “freeze-clamped” before, during, and after NH₄Cl infusion and various intermediates were measured (Table II). Two amino acids, aspartate and alanine, both in relatively low concentration in the liver, were increased 5- to 8-fold after the infusion of 1 mM NH₄Cl and 20- to 30-fold when 2 mM NH₄Cl was infused for 15 min. The glutamate concentration in the perfused liver was approximately doubled by 2 mM NH₄Cl but unaffected at the lower concentration. The concentrations of the adenine nucleotides were not significantly altered at either 1 or 2 mM NH₄Cl. An analysis of glycine (Table II) and of all of the other amino acids (data not shown) in the liver indicated no major changes associated with NH₄Cl infusion.

The high concentrations of both aspartate and alanine in perfused guinea pig liver dropped markedly immediately after cessation of 2 mM NH₄Cl infusion. This decrease corresponded to a marked increase in gluconeogenesis noted after the termination of NH₄Cl (2 mM) shown in Fig. 6, suggesting a direct relationship between the two events. There was a net loss of 6.2 μmol/g of liver from the three major amino acids (glutamate, aspartate, and alanine) observed to change in the 15-min period after cessation of 2 mM NH₄Cl infusion. Negligible amounts of amino acids were released by the liver into the perfusion medium and the slight increase in oxygen consumption noted during this period was not sufficient to account for their subsequent oxidation in the citric acid cycle. This net loss in amino acids could account for 3.1 μmol of glucose and 3.1 μmol of urea/g of liver in 15 min if converted entirely to glucose.

**Table II**

**Effect of NH₄Cl infusion on metabolites from freeze-clamped guinea pig livers perfused with lactate and octanoate**

Livers from 48-hour fasted guinea pigs were perfused as described under Fig. 6. Before NH₄Cl infusion, at the end of its infusion, or 15 min after its cessation, the livers were freeze-clamped and the metabolites were determined as described under “Experimental Procedure.” Values are the means ± standard error for three or four animals.

| Metabolite                | Before NH₄Cl Infusion (μmol/g liver) | During NH₄Cl Infusion (1 mM) (μmol/g liver) | During NH₄Cl Infusion (2 mM) (μmol/g liver) | After NH₄Cl Infusion (1 mM) (μmol/g liver) | After NH₄Cl Infusion (2 mM) (μmol/g liver) |
|---------------------------|--------------------------------------|-------------------------------------------|-------------------------------------------|-------------------------------------------|-------------------------------------------|
| ATP                       | 2.087±0.169                          | 1.900±0.140                               | 2.290±0.092                               | 2.140±0.240                               | 1.84±0.098                               |
| ADP                       | 0.495±0.065                           | 0.562±0.030                               | 0.536±0.044                               | 0.485±0.024                               | 0.451±0.036                               |
| AMP                       | 0.225±0.024                           | 0.386±0.032                               | 0.125±0.010                               | 0.132±0.020                               | 0.157±0.030                               |
| Acetoacetate              | 0.390±0.020                           | 0.405±0.024                               | 0.434±0.012                               | 0.277±0.056                               | 0.396±0.034                               |
| β-hydroxybutyrate         | 0.112±0.007                           | 0.080±0.003                               | 0.085±0.009                               | 0.080±0.012                               | 0.126±0.008                               |
| Aspartate                 | 0.153±0.013                           | 1.210±0.097                               | 3.068±0.068                               | 0.376±0.027                               | 0.50±0.074                                |
| Glutamate                 | 3.040±0.510                           | 4.241±0.437                               | 7.920±1.054                               | 3.471±0.536                               | 4.640±0.320                               |
| Glycine                   | 1.749±0.362                           | 1.617±0.618                               | 1.518±0.538                               | 1.077±0.134                               | 1.145±0.151                               |
| Alanine                   | 0.01±0.002                            | 0.08±0.004                                | 0.47±0.113                                | 0.045±0.006                               | 0.06±0.018                                |
these two major end products by the liver. A calculation of the rate of glucose synthesis during the 15-min period immediately after the cessation of 2 mM NH$_4$Cl infusion indicated that approximately 3 µmol of glucose/g of liver were formed over the depressed level of gluconeogenesis caused by perfusion with octanoate alone. Urea synthesis declined more rapidly than glucose synthesis increased but net urea synthesis during the same 15-min period was approximately 3 µmol/g of liver. The rapid changes in the rates of urea and glucose synthesis noted immediately after removal of NH$_4$Cl complicate the exact calculation of carbon and nitrogen balance but it is probable that the majority of the amino acids stored in the liver are converted to glucose, accounting for the sharp increase in gluconeogenesis.

Further information about the pathway of carbon flow during gluconeogenesis in guinea pig liver is presented in Fig. 7. Octanoate infusion caused the expected decrease in glucose synthesis of about 50% and the combination of 0.2 mM aminoxyacetate, and 2 mM NH$_4$Cl reduced gluconeogenesis to values noted prior to infusion of lactate. Urea synthesis increased after NH$_4$Cl infusion and returned to control levels within 5 min of the termination of NH$_4$Cl infusion. However, the rate of gluconeogenesis did not rebound to the high levels noted after NH$_4$Cl infusion in the absence of aminoxyacetate (see Fig. 6). After 95 min of perfusion, octanoate infusion was terminated and the synthesis of glucose immediately returned to levels approximately equal to those noted before the introduction of aminoxyacetate and NH$_4$Cl. This increase in glucose synthesis reflected the formation of P-enolpyruvate by mitochondrial P-enolpyruvate carboxykinase, a pathway not inhibited by aminoxyacetate (8). An analysis of the concentration of the two amino acids which changed dramatically during NH$_4$Cl infusion, glutamate and aspartate, indicated that glutamate was present in guinea pig liver at a level of 5.20 µmol/g of liver during the infusion with aminoxyacetate and NH$_4$Cl and did not change when the infusion of these two compounds was terminated. Aspartate, on the other hand, dropped from a concentration of 1.35 µmol/g of liver to 0.29 µmol after the infusion of aminoxyacetate and NH$_4$Cl. It is probably that aminoxyacetate, by inhibiting aspartate aminotransferase prevents the buildup of aspartate in the liver. This blocks the normal rebound in gluconeogenesis shown in Fig. 6 after cessation of infusion of 2 mM NH$_4$Cl. The effect of aminoxyacetate did not appear to be complete, however, since the concentration of aspartate in the liver rose from a basal value of 0.153 µmol/g of liver prior to NH$_4$Cl infusion to 1.35 µmol despite the presence of the inhibitor. This increase in aspartate may support the urea cycle which is functioning at an elevated rate despite the inhibition of gluconeogenesis by aminoxyacetate.

**DISCUSSION**

It is well documented that the oxidation of fatty acids by guinea pig liver inhibits gluconeogenesis from a number of substrates. Studies with isolated guinea pig liver mitochondria have demonstrated a close correlation between the control of P-enolpyruvate formation and control of gluconeogenesis in the intact, perfused liver (4, 7, 8, 32). Fatty acid oxidation by isolated guinea pig liver mitochondria markedly diminishes P-enolpyruvate formation, an effect shown to be directly related to the oxidation-reduction state of the mitochondria (Table I). Recently Bryla (28) reported that oleate plus carnitine inhibited P-enolpyruvate synthesis from α-ketoglutarate in rabbit mitochondria. When aspartate aminotransferase was blocked by addition of aminoxyacetic acid, there was a 4-fold stimulation of P-enolpyruvate synthesis from α-ketoglutarate which was markedly diminished by octanoate, oleate, or octanoylcarnitine. These findings offer further support for the role of the NAD$^+$/NADH ratio in modulating the rate of mitochondrial P-enolpyruvate formation.

In this study we offer evidence that the effect of fatty acids on P-enolpyruvate synthesis from either α-ketoglutarate or malate can be reversed by the addition of NH$_4$Cl to the mitochondrial incubation medium. This is presumably due to a displacement of the glutamate dehydrogenase equilibrium toward glutamate synthesis, resulting in an increase in the NAD$^+$/NADH ratio. This is consistent with an increased rate of glutamate formation and release by the mitochondria. Aspartate also increases due to transamination with oxalacetate generated from the substrate malate. It is of interest that the addition of aminoxyacetate acid to the incubation medium together with NH$_4$Cl (in the absence of exogenous fatty acids) resulted in the highest rate of P-enolpyruvate formation noted in this study, but at the same time markedly reduced the level of transamination of oxalacetate to aspartate. It is apparent that, if the level of glutamate is increased by the addition of NH$_4$Cl, aspartate aminotransferase can effectively compete with P-enolpyruvate carboxykinase and divert oxalacetate toward aspartate formation. The rate of transamination of oxalacetate is remarkably constant (see Table I) even when glutamate is increased to very high values by the addition of cycloheximide, an inhibitor of energy transfer at site 1.

The use of cycloheximide with isolated guinea pig liver mitochondria offers further insight into the relationship between the oxidation-reduction state and P-enolpyruvate...
synthesis. Previous studies by Jomain-Baum et al. (17) demonstrated a virtually complete inhibition of hepatic gluconeogenesis by cycloheximide infusion into isolated guinea pig liver. Cycloheximide apparently causes this effect by interacting at site 1 of the respiratory chain, causing an increase in the level of hepatic NADH and a decrease in the concentration of ATP. Cycloheximide added together with octanoate or 1-palmitoyl carnitine markedly reduces the rate of P-enolpyruvate formation and lowers ATP synthesis by isolated guinea pig liver mitochondria. Interestingly, the rate of total ketone body formation from octanoate was stimulated 2-fold by cycloheximide addition, whereas the ratio of acetoacetate to \( \beta \)-hydroxybutyrate by isolated guinea pig liver mitochondria was decreased. Lopes-Cardozo and Van Den Bergh (33) have shown an inverse relationship between the concentration of citric acid cycle intermediates, such as malate, and the synthesis of ketone bodies from palmitate by rat liver mitochondria. They also noted a decrease in the acetoacetate to \( \beta \)-hydroxybutyrate ratio when the concentration of citric acid cycle intermediates was increased.

There have been suggestions in the literature that fatty acids stimulate P-enolpyruvate synthesis in rabbit or guinea pig liver by uncoupling mitochondrial respiration (34-36). In the present study we show no uncoupling of guinea pig liver mitochondria by octanoate at concentrations of 0.5 mM or below. At a higher concentration of octanoate, 1 mM, the mitochondria are loosely coupled. It is known that 2,4-dinitrophenol will stimulate P-enolpyruvate formation from a number of citric acid cycle intermediates but in those experiments ATP was added to the incubation medium. While it is possible that uncoupling of oxidative phosphorylation by fatty acids plays a physiological role in the regulation of hepatic gluconeogenesis, it is an unlikely explanation for the marked decrease in glucose synthesis noted in these papers. Fatty acids are also known to inhibit ATP-ADP exchange as well as alter the kinetics of tricarboxylate carriers (37-41). It is also possible that fatty acids, such as octanoate, might reduce the rate of P-enolpyruvate formation and subsequent efflux from the mitochondria by such a mechanism. However, palmitoylcarnitine, which is one of the least effective fatty acyl derivatives in inhibiting the various tricarboxylate carriers, very effectively decreases P-enolpyruvate synthesis. Our data are, therefore, consistent with Morel et al. (42) who express some reservations concerning the physiological significance of fatty acid inhibition of mitochondrial transporters.

It is known that during starvation the mitochondrial oxidation-reduction state in guinea pig and rabbit liver shifts markedly more oxidized (13, 43). This is determined by freeze-clamping liver from both species and calculating the NAD\(^+\)/NADH ratio by measuring intermediates of either the \( \beta \)-hydroxybutyrate dehydrogenase or the glutamate dehydrogenase reactions. From these studies it was not clear why the mitochondrial oxidation-reduction state should shift more oxidized during starvation since the oxidation of fatty acids by liver mitochondria is generally accelerated by starvation. The present paper shows that ammonia can reverse the effect of fatty acids on the liver and cause an oxidized mitochondrial NAD\(^+\)/NADH ratio. This effect of ammonia is concentration-dependent. At concentrations of 0.5 and 1 mM, \( \text{NH}_3\text{Cl} \) reverses the inhibition of gluconeogenesis normally associated with fatty acid metabolism. However, at an \( \text{NH}_3\text{Cl} \) concentration of 2 mM the stimulation of gluconeogenesis from lactate is not as immediate as it is with lower levels of ammonia. The portal blood ammonia concentration in 48-hour fasted guinea pigs is between 0.7 and 1 mM so that the concentrations of \( \text{NH}_3\text{Cl} \) used in this study are near the physiological level. Furthermore, Garber and Hanson (13) reported an ammonia level of 0.72 mM in the livers of fed guinea pigs which increased to 1.26 mM after 48 hours of fasting.

The steady state concentration of ammonia in the liver represents a balance between the rate of ammonia removal in the urea cycle and its generation by reactions such as glutamate dehydrogenase, glutaminase, and the purine cycle. There is currently some dispute concerning the role of glutamate dehydrogenase in generating ammonia based on the observation of Mendes-Mourão et al. (44) that L-leucine inhibits glutamate dehydrogenase while stimulating urea synthesis. These authors suggest that glutamate dehydrogenase operates in the direction of glutamate synthesis and not for net ammonia generation. However, if the concentration of glutamate is elevated artificially, such as by the perfusion of 2 mM \( \text{NH}_3\text{Cl} \), it is clear that some of the glutamate may be deaminated, supporting urea synthesis. There was a net decrease of 3.3 \( \mu \)mol of glutamate within the 15 min after termination of \( \text{NH}_3\text{Cl} \) (Table II) which could not be accounted for by release into the perfusion medium or by transamination to form other amino acids.

It is possible that during fasting the oxidation-reduction state of guinea pig liver mitochondria can be influenced by the metabolism of ammonia as well as fatty acids. Our liver perfusion experiments using ammonium chloride together with fatty acids demonstrate a potential interaction which can dramatically alter the pattern of regulation of glucose synthesis in the liver. This observation also provides a link between two previously unresolved observations: the oxidized mitochondrial NAD\(^+\)/NADH ratio during fasting and the high rates of fatty acid oxidation which normally characterize starvation. It is probable that the availability of ATP will limit the rate of disposal of ammonia through the urea cycle. Our studies (Fig. 6) indicate a lack of exact stoichiometry between the rate of urea synthesis and the concentration of \( \text{NH}_3\text{Cl} \) in the perfusion medium. Urea synthesis from 2 mM \( \text{NH}_3\text{Cl} \) is not double that found with 1 mM \( \text{NH}_3\text{Cl} \), suggesting some limitation on this process. However, there is no relationship between the rate of gluconeogenesis and ureagenesis. With 2 mM \( \text{NH}_3\text{Cl} \) infusion, glucose synthesis remains at levels noted prior to its infusion and does not increase until \( \text{NH}_3\text{Cl} \) infusion is terminated.

One important aspect of the interaction between ammonia and fatty acid metabolism is the key role of aspartate and, to a lesser extent, alanine as potential reservoirs of carbon for glucose synthesis. When levels of ammonia high enough to temporarily exceed the capacity of the urea cycle were used (2 mM \( \text{NH}_3\text{Cl} \)), the concentrations of aspartate and alanine increased 20- and 30-fold, respectively. The total aspartate concentration was a remarkably high 6 mM (assuming the intracellular water concentration is approximately 50% of total weight). The portal blood ammonia concentration in 48-hour fasted guinea pigs is between 0.7 and 1 mM so that the concentrations of aspartate detectable in the perfusate indicate that this decrease in cellular aspartate is not due to release of the amino acid from the cell. These findings are in general accord with a
previous study reported by Brosnan and Williamson (46) who demonstrated that the administration to rats of 2.5 mmol of NH₄Cl/kg body weight increased the hepatic alanine and aspartate concentrations and also shifted the mitochondrial oxidation-reduction state more oxidized. In this study, cycloserine, an inhibitor of alanine aminotransferase blocked the increase in alanine after NH₄Cl loading, resulting in a further increase in aspartate. The authors concluded that aspartate aminotransferase was quantitatively more important than alanine aminotransferase for removal of ammonia. We also used cycloserine in the perfused guinea pig liver and found it did not prevent the rapid rise in glucose synthesis noted when 2 mM NH₄Cl infusion is terminated (data not shown). Aspartate would, therefore, seem to be quantitatively more important in the rapid rebound in gluconeogenesis observed when NH₄Cl infusion stops and it may be the principal storage form for ammonia and carbon when the ammonia concentration exceeds the capacity for clearance in the urea cycle.

The relationship between gluconeogenesis and the urea cycle is worthy of considerably more study to establish (a) whether competition between the two processes for ATP can limit the rate of either pathway and (b) whether the fumarate generated in the urea cycle can be converted to P-enolpyruvate in the cytosol, thus implicating ureogenesis in the supply of gluconeogenic anions in the cytosol. The degree to which the latter sequence occurs during gluconeogenesis from amino acids has not been accurately assessed. It is possible that the liver can regulate the rate of glucose synthesis relative to ureogenesis by storing excess ammonia as amino acids, such as aspartate and alanine, while using other available substrates for gluconeogenesis. It is clear from the present study that ammonia metabolism can dramatically alter the rate of gluconeogenesis in guinea pig liver. Any proposed mechanisms for the regulation of this pathway should involve an integration of gluconeogenesis with ureogenesis.

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M Jomain-Baum and R W Hanson

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