Rate-limiting Steps for Hepatic Gluconeogenesis

MECHANISM OF OXAMATE INHIBITION OF MITOCHONDRIAL PYRUVATE METABOLISM*

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Oxamate, structural analog of pyruvate, inhibits gluconeogenesis from pyruvate or substrates yielding pyruvate. The inhibitory effect is the result of a decreased mitochondrial pyruvate utilization. Although the inhibition of gluconeogenesis is competitive for pyruvate, in isolated mitochondria oxamate displays a mixed type kinetics inhibitory pattern of pyruvate utilization. Evidence is presented indicating that this mixed type pattern of inhibition is the result of the action of oxamate on two different sites: 1) noncompetitive inhibition of pyruvate carboxylation, and 2) competitive inhibition of pyruvate entry into the mitochondria. At concentrations of pyruvate above 0.4 mM, although pyruvate carboxylation is decreased by 40% by oxamate, no detectable effects on the gluconeogenic flux were observed. This finding strongly indicates that pyruvate carboxylase is not an important rate-limiting step for hepatic gluconeogenesis. Thus, the inhibition of gluconeogenesis at low pyruvate concentrations (<0.4 mM) seems to be the result of an interaction of oxamate with the mitochondrial pyruvate translocator, indicating that pyruvate transport across the mitochondrial membrane is the first nonequilibrium step in the gluconeogenic pathway when low physiological concentrations of this substrate are utilized.

We have recently described (1) that oxamate ($^-$OOC-CO-NH$_2$), structural analog of pyruvate, known by its specific inhibitory effect on lactate dehydrogenase (2, 3) was a potent inhibitor of hepatic gluconeogenesis. This effect was observed from substrates yielding pyruvate other than lactate, ruling out lactate dehydrogenase as the primary site of action of oxamate. The lack of effect in inhibiting gluconeogenesis from substrates whose metabolism does not involve pyruvate carboxylation, points to mitochondrial pyruvate metabolism as the site of oxamate action. Although oxamate has been reported to inhibit purified rat liver pyruvate carboxylase (4), the following indirect evidence strongly supports that its primary site of action in inhibiting gluconeogenesis is related to pyruvate transport across the mitochondrial membrane. 1) Oxamate inhibits gluconeogenesis only from low, physiological (<0.4 mM) concentrations of pyruvate when presumably the pyruvate translocator is limiting (5–8). 2)

The kinetic of oxamate inhibition of gluconeogenesis is entirely different from that reported on isolated purified pyruvate carboxylase. The former is competitive (1), and the latter is noncompetitive (4).

Our current work is a more detailed and direct study of oxamate interaction with the mitochondrial metabolism of pyruvate. Evidence has been obtained, by using a new experimental approach, which supports the conclusion that oxamate inhibits pyruvate transport across the mitochondrial membrane. Pyruvate carboxylation was inhibited by oxamate at supraphysiological concentrations of substrate regardless of whether it was added to the incubation medium or generated intramitochondrically from alanine. Since gluconeogenesis is not inhibited by oxamate at these pyruvate concentrations, this observation adds directly conclusive support to previous evidence suggesting that pyruvate carboxylation is not rate-limiting for gluconeogenesis (1).

EXPERIMENTAL PROCEDURES

Liver Perfusion and Liver Cells Isolation—Livers from starved male Wistar Rats (180–220 g) were perfused with Krebs-Ringer bicarbonate buffer in a flow-through system as previously described (1, 9). Oxygen consumption was measured polarographically with a Clark type electrode. The pO$_2$ in the effluent perfusate under basal conditions varied from 290 to 350 mm Hg. Routinely, the liver was allowed to equilibrate by perfusing it for 30 min in the absence of any substrate.

Liver cells from fasted male Wistar rats were prepared by the collagenase digestion technique (10) modified as in Ref. 1. Incubations (≃20 mg wet wt/ml) were performed in stoppered 25-ml plastic conical flasks at 37 °C. The incubation medium consisted of Krebs-Ringer bicarbonate buffer containing 1.5% gelatin. The medium was equilibrated with a gas mixture containing 95% O$_2$ and 5% CO$_2$.

Preparation and Incubation of Mitochondria—Rat liver mitochondria were isolated by differential centrifugation from fed male Wistar rats (200–250 g) essentially as described by Schneider and Hogeboom (11). The isolation medium contained 225 mM mannitol, 75 mM sucrose and 0.1 mM EDTA, pH 7.4. Respiratory control ratios were routinely measured using a buffered KCl medium and 10 mM glutamate and 1 mM malate as substrates (12), and values were usually greater than 7. The mitochondrial incubation medium used for studying pyruvate metabolism contained 110 mM KCl, 15 mM KHCO$_3$, 20 mM MOPS,[1] 2 mM MgCl$_2$, 5 mM KH$_2$PO$_4$, and 0.5 mM EDTA, pH 7.4. The medium was equilibrated with a gas mixture containing 95% O$_2$ and 5% CO$_2$ prior to addition of mitochondria. The final concentration of mitochondria was 1–5 mg of protein/ml. Protein was determined by a modification of the biuret procedure using crystalline bovine serum albumin as standard (13).

Pyruvate Carboxylation—The rate of pyruvate carboxylation was estimated by measuring the rate of H^{14}CO$_3^-$ (0.06 Ci/mol) incorporation into acid stable metabolites (14, 15) at 28 °C. Incubations (2 ml) containing 5 mg of mitochondrial protein/ml, and different concentrations of pyruvate were performed in 25-ml conical flasks for a period of 15 min. Aliquots of 0.5 ml were taken at 5, 10, and 15 min

[1] The abbreviation used is: MOPS, 3-(*N*-morpholino)propanesulfonic acid.

and immediately acidified with perchloric acid (final concentration 4%). After all the CO_2 had evolved, the samples were transferred to 20-ml vials, mixed with 10 ml of scintillant (Quickscint 1) and the radioactivity determined by liquid scintillation spectrometry. The specific activity of the $H^{14}CO_3^-$ was determined by measuring the radioactivity of an aliquot of $H^{14}CO_3^-$ solution in 100 μl of hyamine hydroxide. The rate of $H^{14}CO_3^-$ incorporation was estimated from the slope of a plot of $H^{14}CO_3^-$ incorporation as a function of time.

Pyruvate Decarboxylation—Rates of CO_2 production from [1-^{14}C] pyruvate were determined at 28 °C. Incubations (1 ml containing 1–5 mg of mitochondrial protein) were performed in 25-ml conical flasks sealed with rubber stoppers from which were suspended plastic cups containing a small piece of flutted filter paper. The incubation medium contained 0.1 μCi/ml of [^{14}C]pyruvate together with 0.2 or 1.0 mM pyruvate and was equilibrated with a gas mixture of 95% O_2 and 5% CO_2 prior to addition of mitochondria. Shortly before the incubation was terminated, 0.3 ml of hyamine hydroxide was injected through the stopper into the center well. After 5, 10, and 15 min of incubation, the reaction was terminated by injection of 0.5 ml of 2 N H_2SO_4 into the medium followed by a further 30-min incubation to allow diffusion of all the CO_2 into the hyamine. The plastic cups were transferred to scintillation vials containing 10 ml of Quickscint 1 to be counted. An aliquot of the medium was counted for determination of the specific radioactivity of the [^{14}C]pyruvate.

Assay of Enzymes and Metabolites—Total pyruvate carboxylase activity was measured in mitochondria lysed by freeze-thawing, using the radioactive $^{14}CO_2$ fixation procedure described by Weinberg and Utter (16). Pyruvate and ATP were omitted from the blanks.

Pyruvate dehydrogenase activity was measured in mitochondria preincubated under the conditions used to measure pyruvate oxidation. After a 5-min preincubation period, the mitochondria suspension was diluted with an equal volume of isolation medium containing 50 mM Tris-HCl, pH 7.0, 5 mM EDTA, 25 mM NaF, 1 mM dithiothreitol, 10% (v/v) rat serum, and 0.5% Triton X-100 at 0 °C and sedimented in an Eppendorf microcentrifuge for 10 s. The supernatant was discarded and the pellet frozen in liquid nitrogen. Just before assay, they were resuspended in isolation medium and assayed at 30 °C as previously described (17). The total activity of pyruvate dehydrogenase was obtained by incubating the sample for 30 min in the presence of 10 mM $MgCl_2$ and 0.4 mM $CaCl_2$ (18).

For the determination of intermediate metabolites, aliquots of mitochondrial suspensions were taken at times given in the legend of figures and tables and acidified with perchloric acid (4% w/v final concentration). The pH was adjusted to 6.0 with 3 N KOH containing 0.5 M triethanolamine. The precipitated $KClO_4$ was removed by centrifugation. Metabolites were measured fluorimetrically or spectrophotometrically, according to the expected concentration of each metabolite, as described elsewhere (19, 20).

Materials—All the reagents were of the highest purity available, most of them obtained from Sigma. Collagenase (174 units/mg) was obtained from Worthington. The enzymes were obtained from Boehringer Mannheim. [1-^{14}C]Pyruvate, specific activity 25.9 mCi/mmol, and [1-^{14}C]bicarbonate (57 mCi/mmol) were obtained from Amersham. [1-^{14}C]Pyruvate was dissolved in water upon arrival, and aliquots containing 2.5 μCi were stored at −20 °C until they were used.

RESULTS

Effect of Oxamate on Hepatic Gluconeogenesis—The results of Fig. 1, in agreement with previous observations (1), indicate that oxamate is a potent inhibitor of hepatic gluconeogenesis. It is shown how the inhibition of glucose production induced by oxamate in the isolated perfused liver becomes progressively smaller as the pyruvate concentration increases. An inverse relationship between oxamate inhibition of glucose output from alanine or lactate and pyruvate availability was also found when isolated liver cells were used (results not shown). So, regardless of the experimental model or substrate utilized, it is evident that oxamate acts as a competitive inhibitor for gluconeogenesis from pyruvate.

Effect of Oxamate on Rates of Pyruvate Utilization by Rat Liver Mitochondria—Fig. 2 is a plot of rates of pyruvate utilization *versus* pyruvate concentration with or without 2 mM oxamate. Oxamate inhibits pyruvate utilization over the whole range of pyruvate concentrations tested. Further char-
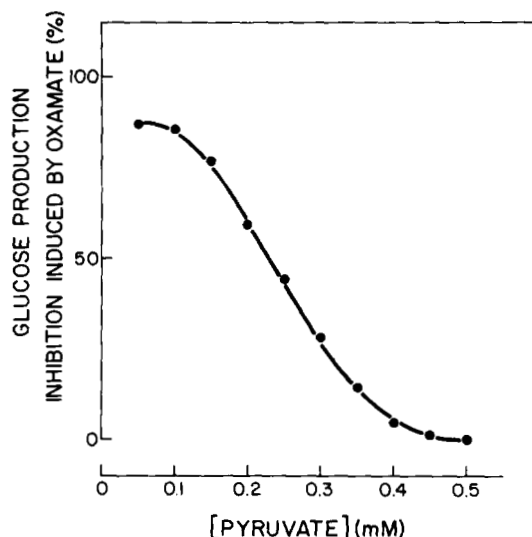

FIG. 1. **Influence of pyruvate concentration on the inhibitory effect of oxamate on gluconeogenesis in isolated perfused liver.** Rat livers from starved rats were perfused as described in the "Experimental Procedures" section in the presence and in the absence of 0.2 mM oxamate. Pyruvate supply was increased at a constant rate by using a gradient former. The outflow pO_2 was continuously monitored and perfusate samples collected in 2–3 min intervals.
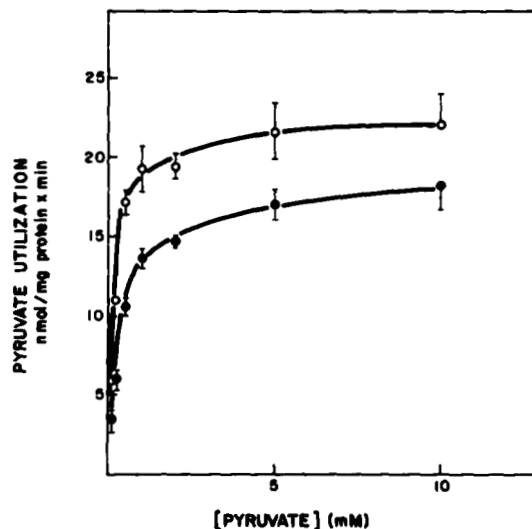

FIG. 2. **Effect of oxamate on rates of pyruvate utilization by rat liver mitochondria.** Isolated mitochondria (3–5 mg of protein/ml) were incubated as described in the "Experimental Procedures" section for 5, 10, and 15 min in the absence (O) or in the presence (●) of 2 mM oxamate. Initial substrate concentrations were as indicated in the Figure. Values shown are means for four to six experiments ± S.E.

acterization of the oxamate effect was carried out by titrating pyruvate utilization in the presence of increasing concentrations of the inhibitor. A reciprocal plot of these data (Fig. 3) gives straight lines intersecting above $1/x$ axis, indicating a mixed type inhibition pattern. The apparent K_i for oxamate was calculated from the replot of the slopes of lines in Fig. 3 *versus* oxamate concentration giving a value of 1.6 mM. This value is of the same order of magnitude required to produce 50% inhibition of gluconeogenesis from lactate in isolated hepatocytes (1). This finding fully supports our previous hypothesis that oxamate inhibited hepatic gluconeogenesis by restricting mitochondrial pyruvate metabolism (1).

Effect of Oxamate on Mitochondrial Metabolism of Pyruvate—Table I shows the effect of oxamate on the fate of

pyruvate utilized by isolated mitochondria. Whether low, 0.2 mM or near saturating concentrations of pyruvate were utilized, oxamate proportionally decreased all the metabolic conversions of pyruvate. In other words, the percentage of pyruvate carbon accounted for by CO_2 production or carboxylating products, malate plus citrate, was maintained constant regardless of the degree of pyruvate utilization. This observation is compatible with an oxamate effect restricting pyruvate entry into the mitochondria. The possibility that oxamate inhibited simultaneously pyruvate dehydrogenase as well as pyruvate carboxylase has been studied by measuring the activity of both enzymes in mitochondrial lysates. As can be seen in Table II, oxamate inhibited the activity of pyruvate carboxylase by 40%. Inhibitor titration experiments demonstrated that, in agreement with a previous report on purified enzyme (4), the inhibition was noncompetitive. In contrast, oxamate did not change the activity of pyruvate dehydrogenase nor the proportion of the enzyme present in its active

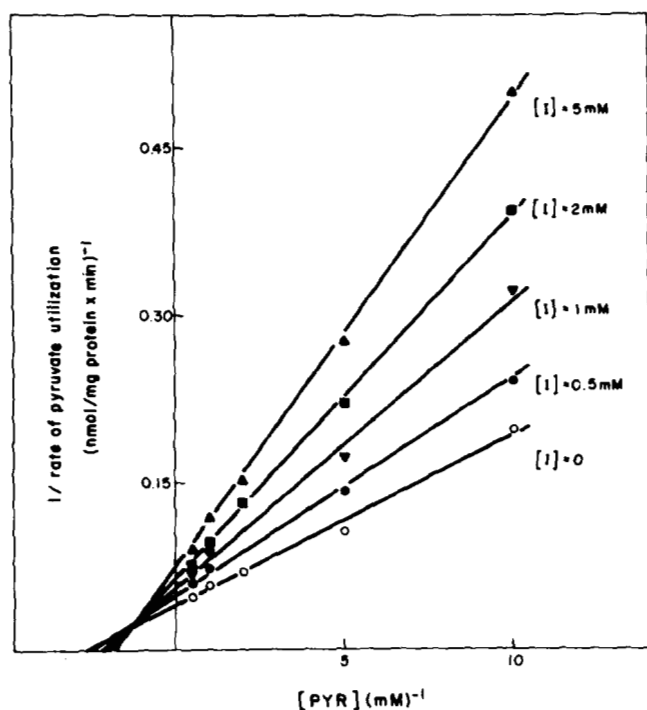[PYR](mM)⁻¹

FIG. 3. **Effect of increasing oxamate concentrations on mitochondrial pyruvate utilization.** Isolated mitochondria were incubated as described in the "Experimental Procedures" section and in the legend to Fig. 2. Values shown are means of duplicate analysis using two different mitochondrial preparations.

form. Thus, the decreased rates of pyruvate decarboxylation in the presence of oxamate can only be understood if this inhibitor acted decreasing the intramitochondrial supply of pyruvate. An alternative explanation could be that a tight coupling might exist between both processes, carboxylation and decarboxylation. In order to clarify if oxamate is able to perturb mitochondrial pyruvate entry besides inhibiting pyruvate carboxylase activity, the effects of oxamate on both processes, pyruvate entry and its carboxylation, have been studied separately.

Effect of Oxamate on Rates of Mitochondrial Pyruvate Carboxylation—Liver mitochondria are known to display significant capacity for transaminating alanine (21–23). We took advantage of this property to generate intramitochondrially pyruvate by supplementing the incubation medium with alanine plus α-ketoglutarate. This experimental design allows the study of the effect of oxamate on pyruvate carboxylation in the absence of eventual undesirable effects of the inhibitor on pyruvate transport. Fig. 4A shows the rates of [¹⁴C]bicarbonate fixation by isolated mitochondria as a function of pyruvate concentration. The reciprocal plot in the *insert* shows a mixed type kinetics inhibitory pattern of oxamate similar to the one observed in Fig. 3 for pyruvate utilization. In contrast, when rates of bicarbonate fixation were determined in mitochondria generating pyruvate intramitochondrially (Fig. 4B), a noncompetitive pattern of inhibition was observed. This observation allows us to conclude that the mixed type kinetics inhibition of mitochondrial pyruvate utilization by oxamate can be dissected away into two different components: 1) a noncompetitive inhibition of pyruvate carboxylation, and 2) a competitive action on pyruvate entry into the mitochondria.

The rate of CO_2 fixation in the presence of alanine as substrate is lower than the rate of CO_2 fixation by exogenously added pyruvate. However, the possibility that oxamate could affect alanine transport and/or transamination can be ruled out based on the following observations:

The rate of pyruvate production in mitochondria incubated with several concentrations of alanine plus α-ketoglutarate in a HCO_3^- deficient buffer was not altered by the presence of oxamate while, under carboxylation conditions, oxamate induced an appreciable accumulation of pyruvate secondary to a decreased flux of alanine-derived pyruvate to oxaloacetate (results not shown). On the other hand, the rate of alanine transamination exceeded the rate of alanine-derived pyruvate carboxylation, suggesting that under these experimental conditions pyruvate supply was not limiting the flux through pyruvate carboxylase (results not shown). A possible explanation for the observed decreased rate of CO_2 fixation in the

TABLE I

Effects of oxamate on mitochondrial pyruvate metabolism

Isolated mitochondria (2–5 mg of protein/ml) were incubated as described in "Experimental Procedures" section for 5–15 min in the absence and in the presence of 2 mM oxamate. Initial substrate concentration was 0.2 and 1 mM pyruvate, respectively. Values shown are means of four to six experiments ± S.E.

	0.2 mM Pyruvate		1 mM Pyruvate	
	Control	Oxamate	Control	Oxamate
	nmol/mg protein × min		*nmol/mg protein × min*	
Pyruvate utilization	10.34 ± 0.55	6.36 ± 0.47	19.20 ± 1.1	14.9 ± 0.99
Pyruvate decarboxylation	2.34 ± 0.09	1.64 ± 0.09	2.85 ± 0.16	2.16 ± 0.09
Pyruvate carboxylation	5.31 ± 0.22	2.39 ± 0.22	9.76 ± 1.1	5.8 ± 0.5
Malate production	3.75 ± 0.38	1.74 ± 0.07	6.21 ± 0.39	4.25 ± 0.5
Citrate production	1.80 ± 0.18	0.95 ± 0.09	2.44 ± 0.28	1.95 ± 0.2
% of pyruvate used accounted for as CO_2	23	25	15	14
% of pyruvate used accounted for as malate	36	35	32	28
% of pyruvate used accounted for as citrate	17	15	13	13

TABLE II

Effects of oxamate on the activities of pyruvate carboxylase and pyruvate dehydrogenase in mitochondrial extracts

Pyruvate carboxylase and pyruvate dehydrogenase activities were measured in mitochondria lysed by freeze-thawing as described in the "Experimental Procedures" section. The concentration of pyruvate in the assay mixture was 1 mM and when present, the oxamate concentration was 2 mM. Values shown are means of at least three experiments ± S.E. Total activity of pyruvate dehydrogenase was $8.4 ± 0.2$ nmol/mg of protein × min.

| | Pyruvate carboxylase | | Pyruvate dehydrogenase | |
	Activity	Type of inhibition[a]	Activity	Fraction of total activity
	nmol/mg protein × min		*nmol/mg protein × min*	*%*
Control	$18.7 ± 3$		$4.5 ± 0.2$	53
Oxamate	$11.3 ± 2$	Non competitive	$4.4 ± 0.4$	52

[a] Determined by the Lineweaver-Burk plot, in which the concentration of pyruvate was varied from 0 to 2 mM and the concentration of oxamate from 0 to 5 mM.
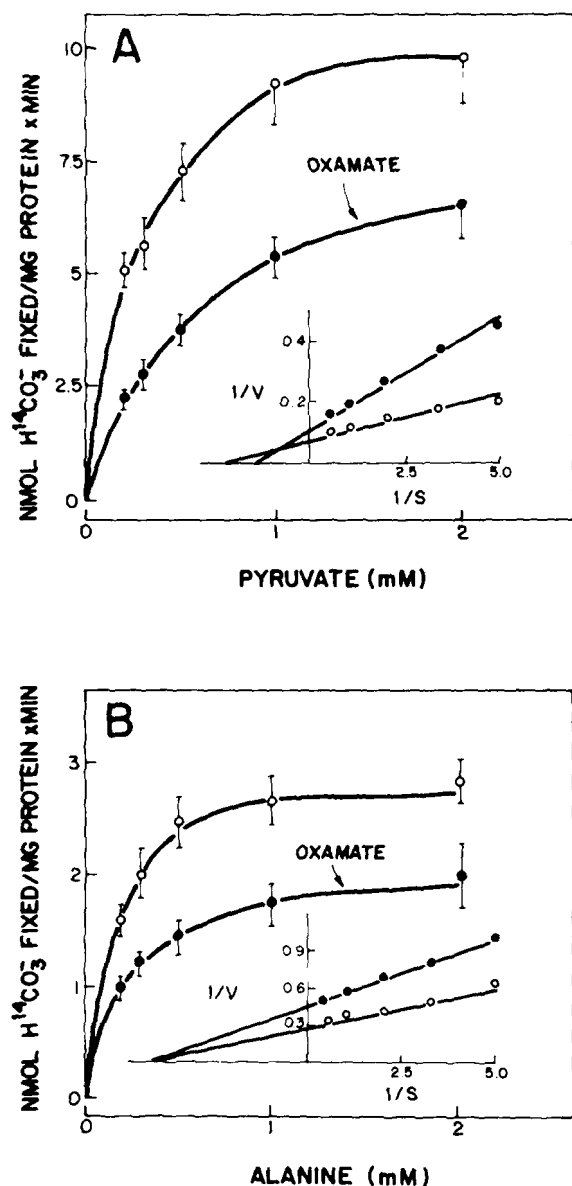

PYRUVATE (mM)

ALANINE (mM)

FIG. 4. **Effect of oxamate (●) on rates of carboxylation of pyruvate externally added or generated intramitochondrially from L-alanine plus α-ketoglutarate.** Mitochondria ($≈2$ mg of protein/ml) were incubated as described in the "Experimental Procedures" section in the presence of $H^{14}CO_3^-$ (0.06 $\mu Ci/\mu mol$) and variable concentrations of pyruvate (*A*) or alanine plus α-ketoglutarate (*B*). All values have been corrected for CO_2 incorporated in the absence of substrates. Values shown are means of four to six experiments ± S.E.

presence of alanine plus α-ketoglutarate might be found in the accumulation, in these conditions, of glutamate, an effective inhibitor of pyruvate carboxylase activity (25).

Effect of Oxamate on Mitochondrial Transport of Pyruvate— The reliability of direct methods for measuring mitochondrial transport of pyruvate is questionable if one takes into account the rather large variation in the kinetic values reported in the literature (see Ref. 26 for a review). We have been unable to obtain meaningful and reproducible results by using methods previously described (24, 27). For this reason the ability of oxamate to inhibit pyruvate transport has been determined by two different procedures. In the first approach, use was made of the noncompetitive nature of the inhibition of pyruvate transport by α-cyano-4-hydroxycinnamate. As more inhibitor is added, pyruvate transport will become rate-limiting for pyruvate metabolism. These inhibitor-titrations have been used in several laboratories in the search for localization of rate-limiting steps in a given metabolic pathway (7, 28). By using this experimental approach, at an extramitochondrial concentration of pyruvate 0.2 mM, linear Dixon plots were obtained. The rate of pyruvate transport was calculated to be 9.52 and 6.6 nmol/mg of protein × min in control- and oxamate-treated mitochondria, respectively. These values are similar to the rates of pyruvate utilization determined at the same extramitochondrial concentration of pyruvate (Table I). According to these calculations, the effect of oxamate in decreasing the rate of pyruvate carboxylation could be accounted for only by its limiting effect on pyruvate supply.

An entirely different and novel approach used for estimating pyruvate transport consisted in measuring rates of alanine formation from externally added equimolar pyruvate and glutamate. Fig. 5, *a* and *b* shows the effect of oxamate on rates of alanine production as a function of pyruvate plus glutamate concentrations. It can be appreciated how, when low concentrations of pyruvate were used, oxamate decreased significantly the rate of alanine production. The reciprocal plot of these data (Fig. 5c) shows that oxamate acted by increasing the apparent K_m of the process without altering significantly the apparent V_{max}. Since no other pyruvate-utilizing reaction was taking place, it seems plausible to conclude that this effect of oxamate is a consequence of its interaction with the pyruvate translocator. In contrast, when higher concentrations of pyruvate were used (Fig. 5, *b* and *d*) no effect of oxamate on rates of alanine production could be detected. The high K_m obtained at concentrations of pyruvate above 0.5 mM with most probability reflects the affinity of the intramitochondrial alanine aminotransferase for this substrate.

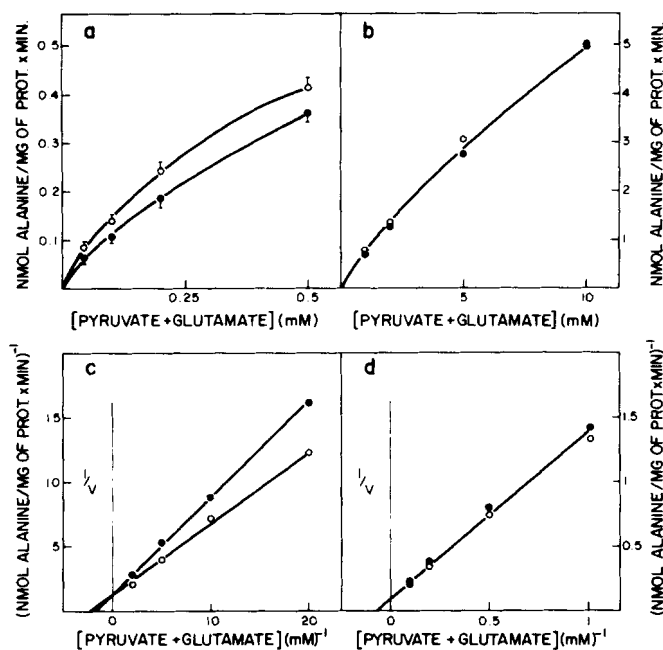Addition of α-cyano-4-hydroxycinnamate (0.1 mM) to the incubation medium fully prevented alanine production, show-

FIG. 5. **Effect of oxamate (●) on rates of alanine production from pyruvate in isolated mitochondria.** Isolated mitochondria (≈ 2 mg of protein/ml) were incubated for 5 and 10 min in a HCO_3^- deficient buffer containing 110 mM KCl, 30 mM Tris, 20 mM MOPS, 2 mM $MgCl_2$, 5 mM KH_2PO_4, and 0.5 mM EDTA, pH 7.4 in the presence of 2 mM arsenite and pyruvate plus glutamate at the concentrations indicated. Values shown in *panel a* are the means of at least three experiments ± S.E.; values shown in *panel b* are means of duplicate samples using two separate preparations of mitochondria.

ing that alanine formation is dependent on pyruvate transport (results not shown). The rates of transport determined by this procedure are lower than calculated above from the inhibitor-titration experiments. Several explanations may account for this observation: 1) Since pyruvate metabolism was prevented, this finding might indicate that maximal rates of pyruvate transport cannot be attained unless intramitochondrial pyruvate is efficiently removed. 2) Since glutamate concentrations were much lower than its normal intramitochondrial concentration (29), it could be that its availability was limiting to maintain effective rates of transamination. 3) Finally, it should be considered that alanine aminotransferase activity was limiting. The latter possibility does not seem to be probable since the reported activity of the transferase (21, 22, 30) is one order of magnitude above the calculated rate of pyruvate transport. At present all these possibilities are being explored experimentally in order to validate this procedure as a quantitative method for measuring mitochondrial transport of pyruvate. So far, it seems reasonable to accept this procedure as a qualitative method to detect perturbations on the pyruvate translocator function. The apparent K_i for oxamate inhibition of pyruvate transport calculated by this procedure was 2.1 mM which is agreeable with the concentration of oxamate required for half-maximal inhibitory effects on both gluconeogenic flux in isolated hepatocytes (1) and pyruvate utilization by intact mitochondria (Fig. 3). The similar apparent inhibition constants on these processes fully supports that the primary site of oxamate action is on the pyruvate translocator. This interpretation finds also support in the fact that the reported K_i of oxamate (200 μM) for the purified rat liver pyruvate carboxylase (4) is almost one order of magnitude lower than that observed in intact mitochondria. This observation indicates that oxamate is not equilibrated across the

mitochondrial membrane suggesting that its entry is a carrier-mediated process probably sharing the pyruvate translocator.

DISCUSSION

Rate-limiting Steps for Gluconeogenesis from Substrates Yielding Pyruvate—There is a great deal of controversy regarding the rate-limiting steps in the gluconeogenic pathway. A common finding when the pathway is stimulated is a "crossover" between pyruvate and phosphoenolpyruvate (31–34). Pyruvate carboxylase and/or phosphoenolpyruvate carboxykinase have been claimed to be the steps activated when the gluconeogenic pathway was stimulated. On the other hand, there are several reports in the literature suggesting that the stimulation of gluconeogenesis by glucagon could be accounted for by inhibition of pyruvate kinase (35, 36). The position of pyruvate carboxylase in the beginning of the pathway make this step a logical important candidate for regulating the gluconeogenic flux. In support of this possibility are the observations of Adams and Haynes (37) that glucagon stimulation of gluconeogenesis coincides with increased mitochondrial pyruvate metabolism and that of Chan *et al.* (23) demonstrating a direct stimulation of pyruvate carboxylase by glucagon. Nevertheless, some authors, in view of the lack of variations in its activity under various nutritional states, claimed for pyruvate carboxylase an anaplerotic role unrelated to the regulation of gluconeogenic flux (38). The observation that pyruvate carboxylase activity far exceeds the capacity of the gluconeogenic pathway (1) casts serious doubts about its regulatory role. The data presented in Figs. 1, 2, and 4 seem to provide firm experimental support to this point of view. At concentrations of pyruvate 0.5 mM and above, oxamate does not significantly inhibit gluconeogenesis (Fig. 1); however, pyruvate carboxylation was reduced by 40% (Fig. 4). This finding implies that some other step(s) must be activated when pyruvate metabolism, and thus gluconeogenesis, is increased despite the concomitant decrease of pyruvate steady state levels, as it is observed in starvation (39) or after glucagon administration (29, 34). The obvious limiting step susceptible of regulatory control seems to be pyruvate transport across the mitochondrial membrane. Reports from several laboratories (5–8, 40–42) seem to indicate that activity of the monocarboxylate translocator may be an important limiting step for mitochondrial pyruvate metabolism and consequently for gluconeogenesis from low (<0.4 mM), presumably physiological, concentrations of this substrate. The rate of transport calculated from the inhibitor-titration experiments (9.5 nmol × mg of protein^{-1} × min^{-1}), taking into account a mitochondrial protein concentration of 51 mg × g^{-1} of liver (43) and correcting for the incubation temperature difference gives a rate of transport of 218 μmol × 100 g^{-1} body weight × h^{-1} which compares very closely with twice the rate of gluconeogenesis from 0.2 mM pyruvate (70–90 μmol × 100 g^{-1} body weight × h^{-1}) (1).

Coupling between Pyruvate Carboxylation and Decarboxylation—Products of pyruvate dehydrogenase activity like acetyl-CoA or ATP are substrates or effectors of the carboxylase. On the other hand, the rate of oxaloacetate formation determines the rate of removal of acetyl-CoA to form citrate and free CoA, preventing end product inhibition of the dehydrogenase. Although a reciprocal control on both activities should be expected, the question of whether a compulsory link exists between both activities is a matter of controversy (44, 45). A recent careful study (46) carried out in isolated liver cells reports a value of 1.9 for the ratio of flux through pyruvate dehydrogenase/pyruvate carboxylase in hepatocytes from fed rats. From data in Table I, as well as from others (47) using

isolated mitochondria, this ratio gives values lower than the unity. This finding probably indicates that the energy demand to maintain cellular functions determines somehow the intramitochondrial partitioning of pyruvate between both reactions. Despite pyruvate dehydrogenase activation by increasing pyruvate availability (48–50), the ratio of pyruvate dehydrogenase/pyruvate carboxylase fluxes decreased when pyruvate concentration was raised from 0.2 to 1 mM in isolated mitochondria (Table I) from 0.44 to 0.29. This observation indicates that pyruvate decarboxylation is saturated at much lower concentrations of pyruvate than carboxylation. Thus, the significance of the reported activation of pyruvate dehydrogenase by increasing pyruvate availability resulting in no net increase in flux is not apparent. The addition of oxamate raised the ratio of flux pyruvate dehydrogenase/pyruvate carboxylase from 0.44 to 0.68 and from 0.29 to 0.37 at 0.2 and 1 mM pyruvate, respectively (Table I). This shift in the partitioning of pyruvate is consistent with the action of oxamate limiting pyruvate supply and inhibiting carboxylation at low (0.2 mM) pyruvate concentration. However, at 1 mM pyruvate oxamate does not inhibit its mitochondrial entry (Fig. 5) and pyruvate dehydrogenase is not inhibited (Table II); nevertheless, the ratio of flux pyruvate dehydrogenase/pyruvate carboxylase increased only slightly, meaning that pyruvate decarboxylation decreased parallel to carboxylation (Table I). At these concentrations of pyruvate, oxamate does not inhibit gluconeogenesis or any other biosynthetic processes (Fig. 1) to account for a decreased energy demand. Thus, the decreased pyruvate oxidation seems to support that a tight compulsory link exists between both pyruvate metabolizing activities.

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