A CARBONIC ANHYDRASE REQUIREMENT FOR THE SYNTHESIS OF GLUTAMINE FROM PYRUVATE IN THE CHAMELEON

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Received June 16,1975

Summary: Large amounts of glutamine were formed in chameleon liver from injected pyruvate or oxaloacetate via reactions which did not involve the citric acid cycle, and which required carbonic anhydrase. Glutamine was increased by injection of dipyruvate (γ-methyl, γ-hydroxy, α-ketoglutarate), but not by injection of glutamate, α-ketoglutarate, isocitrate, succinate, fumarate or malate. Glutamate and α-ketoglutarate increased glutamine if NH_4HCO_3 was added, but carbonic anhydrase was not required. Adding NH_4HCO_3 or carbamyl phosphate doubled the glutamine yield from pyruvate in reactions which required carbonic anhydrase. Dipyruvate may be an intermediate in the pyruvate pathway. Both pyruvate and dipyruvate formed glutamine in liver slices if NH_4HCO_3 was added.

Although two species of reptiles did not form glutamine in the liver *in vivo* from injected glutamate or α-ketoglutarate (1-5), it was formed readily from injected oxaloacetate or pyruvate even without added nitrogen (1-5). Neither [^{14}C]-α-ketoglutarate nor [^{14}C]-glutamate labeled liver glutamine in caimans (5) or chameleons (3, 5), but [^{14}C]-pyruvate did.

Further evidence presented here indicates that glutamine is synthesized in chameleon liver *in vivo* by two pathways. One, which uses glutamate or α-ketoglutarate requires exogenous nitrogen, while the other, involving pyruvate, requires no exogenous nitrogen and is blocked by carbonic anhydrase inhibitors.

Materials and Methods: Chameleons (*Anolis carolinensis*) weighing about 6 g were captured locally and fasted 24 hr before use. Isotonic solutions of Na pyruvate, Na_2 oxaloacetate, Na_2 α-ketoglutarate, and Na_2 glutamate were injected I.P. at a dosage of 30 mMoles/kg; Na_3 isocitrate, Na_2 succinate, Na_2 fumarate, and Na_2 malate were injected at a dosage of 20 mMoles/kg. Dipyruvate (γ-methyl, γ-hydroxy, α-ketoglutaric acid) was given in a dosage of 20 mMoles/kg but was neutralized with 1.5 mMoles $NaHCO_3$/mMole dipyruvate before injection (pH = 6.5). Exogenous nitrogen as Li_2 carbamyl phosphate

or NH_4HCO_3 was toxic if given in large doses and was therefore administered in two injections, 2.5 mMoles/kg at 0 hr and 2.5 mMoles/kg at 45 minutes. After injection the animals were maintained at 28° for various intervals according to the following schedule determined in part by prior knowledge of the rate of catabolism of each compound(1): carbamyl phosphate, NH_4HCO_3, pyruvate + carbamyl phosphate, pyruvate + NH_4HCO_3, dipyruvate + carbamyl phosphate, dipyruvate + NH_4HCO_3 - 1.25 hr; oxaloacetate - 1.50 hr; glutamate + carbamyl phosphate, glutamate + NH_4HCO_3, α-ketoglutarate + carbamyl phosphate, α-ketoglutarate + NH_4HCO_3 - 1.75 hr; pyruvate, isocitrate, succinate, fumarate, malate - 2 hr; glutamate, α-ketoglutarate, dipyruvate - 4 hr. The animals were then killed, their livers removed, quick-frozen, homogenized, and analyzed by methods described previously (3).

Carbonic anhydrase was inhibited by injection of dichlorphenamide (Daramide^R, Merck, Sharpe, and Dohme), 50 mg/kg, or acetazoleamide, (Diamox^R, Lederle Laboratories), 50 mg/kg, 14 hrs before any of the other compounds.

In experiments with tissue slices, about 0.1 g of liver was sliced and placed in 20 ml screw-cap flasks containing Krebs'-Ringer bicarbonate solution. Thirty five micromoles of pyruvate or dipyruvate were added with 35 micromoles of NH_4HCO_3, the flasks were gassed with 95% O_2 - 5% CO_2, capped, and shaken at 40 cycles/min at 30° for 2 hours. Nine volumes of ethanol were then added to stop the reaction, the liver tissue was macerated, the slurry centrifuged, and an aliquot of the supernatant placed on the amino acid analyzer column. In some experiments, 50 mg of dichlorphenamide were added to the flask to block carbonic anhydrase.

The authenticity of the glutamine peak was determined by collecting the effluent responsible for the apparent glutamine peak, heating with 2 N HCl at 100° for 4 hrs, and re-chromatographing. The disappearance of the glutamine peak and the increase in glutamic acid on the amino acid analyzer chromatogram was considered a reliable indication that the peak was indeed glutamine.

The conjugate of pyruvic acid, dipyruvate, was synthesized by adding 2 ml of 12 N HCl to 100 g of pyruvic acid and allowing it to stand for several weeks at room temperature. The colorless crystals derived melted at 116° and in all respects resembled the product prepared by de Jong (6).

Results and Discussion:

Glutamate, α-ketoglutarate, succinate, fumarate, malate, and isocitrate did not increase liver glutamine, while pyruvate, oxaloacetate, and dipyruvate caused a 20 to 40 fold increase (Table 1). Adding NH_4HCO_3 to glutamate or α-ketoglutarate resulted in considerable glutamine synthesis, but if the exogenous nitrogen was supplied by carbamyl phosphate, total glutamine synthesized did not exceed that found after carbamyl phosphate alone.

Glutamine yields were increased greatly by the addition of NH_4HCO_3 to pyruvate or dipyruvate, and by the addition of carbamyl phosphate to pyruvate. Carbamyl phosphate did not increase the yield of glutamine from

Table 1. LIVER SYNTHESIS OF GLUTAMINE, GLUTAMATE, AND ALANINE BY INTACT CHAMELEONS, mMOLES/Kg

Single Compounds					Nitrogen Added					Carbonic Anhydrase Inhibited				
No.	*Cmpd.**	*Gln*	*Glu*	*Ala*	*No.*	*Cmpd.*	*Gln*	*Glu*	*Ala*	*No.*	*Cmpd.*	*Gln*	*Glu*	*Ala*
17	Control	0.20 ±0.06†	1.03 ±0.68	0.40 ±0.26	-	----	----	----	----	2	Control	0.26	0.45	0.50
19	Glu	0.33 ±0.19	---- ----	0.38 ±0.29	5	Glu,NH_4^+	9.98 ±3.42	---- ----	0.97 ±0.15	5	Glu,NH_4^+	9.99 ±3.11	---- ----	4.58 ±1.62
					5	Glu,CAP	2.11 ±0.47	---- ----	0.74 ±0.15	-	----	----	----	----
16	αKG*	0.29 ±0.18	2.56 ±1.07	0.26 ±0.17	5	$\alpha KG,NH_4^+$	4.91 ±2.18	4.06 ±1.54	1.24 ±1.22	5	$\alpha KG,NH_4^+$	5.79 ±2.13	2.70 ±1.41	3.10 ±1.27
					4	αKG,CAP	2.29 ±1.55	2.37 ±1.30	0.87 ±0.79	-	----	----	----	----
13	OxAc*	4.09 ±3.53	2.40 ±0.93	2.55 ±1.47	-	----	----	----	----	4	OxAc	0.32 ±0.23	1.15 ±0.35	0.92 ±0.41
1	ICit*	0.32	1.33	0.38	-	----	----	----	----	-	----	----	----	----
5	Suc*	0.16 ±0.06	0.93 ±0.49	0.45 ±0.28	-	----	----	----	----	-	----	----	----	----
5	Fum*	0.14 ±0.05	0.99 ±0.45	0.66 ±0.26	-	----	----	----	----	-	----	----	----	----
5	Mal*	0.15 ±0.08	0.70 ±0.67	0.40 ±0.20	-	----	----	----	----	-	----	----	----	----
5	CAP*	3.62 ±1.65	1.00 ±0.24	1.60 ±0.62	-	----	----	----	----	5	CAP	5.02 ±0.80	0.85 ±0.21	2.62 ±0.63
5	NH_4^+*	0.42 ±0.31	1.30 ±0.47	0.23 ±0.05	-	----	----	----	----	-	----	----	----	----
18	Pyr*	7.38 ±4.71	3.65 ±0.96	4.23 ±1.58	-	----	----	----	----	10	Pyr	0.47 ±0.39	0.59 ±0.16	4.64 ±2.43
					7	Pyr,CAP	14.13 ±3.37	1.29 ±0.48	5.62 ±1.69	4	Pyr,CAP	0.42 ±0.22	0.89 ±0.31	5.25 ±2.22
					5	Pyr,NH_4^+	12.24 ±2.80	2.35 ±1.10	4.74 ±0.78	5	Pyr,NH_4^+	3.80 ±1.52	1.05 ±0.25	9.05 ±2.83
12	DiP*	5.20 ±1.95	2.11 ±0.66	0.46 ±0.24	-	----	----	----	----	8	DiP	5.45 ±3.66	1.24 ±0.47	1.67 ±0.69
					14	DiP,CAP	6.39 ±3.41	2.18 ±1.10	1.79 ±1.40	6	DiP,CAP	5.43 ±1.06	1.05 ±0.32	3.39 ±2.33
					14	DiP,NH_4^+	11.16 ±3.37	3.25 ±1.54	0.75 ±0.63	8	DiP,NH_4^+	5.20 ±1.33	0.92 ±0.33	2.45 ±1.52

* Cmpd. = compound injected, αKG = α-ketoglutarate, OxAc = oxaloacetate, ICit = isocitrate Suc = succinate, Fum = fumarate, Mal = malate, CAP = carbamyl phosphate, Pyr = pyruvate, DiP = "dipyruvate" = γ-methyl, γ-hydroxy, α-ketoglutarate, NH_4^+ = NH_4HCO_3; † s.d.

dipyruvate. Ammonium bicarbonate alone increased liver glutamine very little.

Carbonic anhydrase inhibition by either acetazoleamide or dichlorphenamide blocked the formation of glutamine from oxaloacetate, pyruvate, and pyruvate plus carbamyl phosphate, and greatly reduced the yield from pyruvate plus NH_4HCO_3, but it had no effect on glutamine synthesis from dipyruvate, from carbamyl phosphate alone or from glutamate or α-ketoglutarate to which NH_4HCO_3 had been added. If we assume that the latter reactions are the classical ones for glutamine synthesis, then these reactions do not require carbonic anhydrase. In general, inhibition of carbonic anhydrase seemed to increase liver alanine. Oxaloacetate and pyruvate were good precursors of alanine, while the dimer was not, suggesting that dipyruvate does not break into two molecules of pyruvate.

The chemical nature of the dimer of pyruvic acid inside the cells is unknown. When synthesized chemically, it has four possible structures - two as isomeric acids and two as isomeric lactones. If only one species is active in glutamine synthesis, then the compound is even more active than it appeared.

Liver slices. Table 2 shows the effect of pyruvate and dipyruvate on glutamine synthesis in liver slices. In the experiment in vitro, transamination of pyruvate to alanine was favored over glutamine synthesis. The stability of dipyruvate was again manifest by its negligible effects on alanine production. Inhibition of carbonic anhydrase partially blocked the synthesis of glutamine from pyruvate without affecting the synthesis from dipyruvate. The liver slice experiments were not as definitive as those performed in vivo.

Evidence that dipyruvate is synthesized in vivo from pyruvate in reptiles and that it is a direct precursor of glutamine is insufficient. The compound has been isolated in certain plants by Linko and Virtanen (8) and pyruvate was also shown to be a precursor of glutamine, but their evidence

Table 2. SYNTHESIS OF GLUTAMINE, GLUTAMIC ACID, AND ALANINE IN CHAMELEON LIVER SLICES (mMoles/kg liver/hr)

No.	*Substrate*	*Gln*	*Glu*	*Ala*
6	Control (Krebs'-Ringer only)	0.32 *±0.29*	1.13 *±0.17*	0.77 *±0.38*
4	NH_4HCO_3	0.27 *±0.07*	1.23 *±0.66*	0.73 *±0.61*
16	pyruvate + NH_4HCO_3	5.00 *±2.72*	1.10 *±0.58*	10.83 *±2.67*
9	pyruvate + NH_4HCO_3 + dichlorphenamide	1.40 *±1.09*	1.72 *±1.32*	12.80 *±5.62*
4	dipyruvate + NH_4HCO_3	2.52 *±1.38*	0.78 *±0.29*	0.34 *±0.11*
4	dipyruvate + NH_4HCO_3 + dichlorphenamide	3.16 *±1.09*	0.62 *±0.17*	1.20 *±1.06*

does not permit the conclusion that pyruvate is conjugated to the dimer in the plant. They concluded that glutamate was derived from glutamine but that glutamate did not produce glutamine. Örström, et al. (7) noted that glutamine was formed from pyruvate and oxaloacetate but not from glutamate or α-ketoglutarate in liver slices of birds. Whether the pathways observed by them are the same as those reported here for reptiles has not been determined but the data suggests that they are at least similar.

Although the reaction sequence is uncertain, the evidence is strong that at least two routes of glutamine synthesis are utilized in the liver of the chameleon, and that the route from pyruvate to glutamine may involve the synthesis of dipyruvate or some closely related compound. If dipyruvate is an intermediate, carbonic anhydrase appears to be involved in its synthesis from pyruvate. Carbonic anhydrase may act at a site associated with nitrogen transfer, however, since glutamine synthesis from dipyruvate and NH_4HCO_3 is partially inhibited by dichlorphenamide.

The role of carbamyl phosphate in glutamine synthesis from pyruvate is

uncertain, but apparently is not identical to that played by NH_4HCO_3 in these reactions. It was at least as good as NH_4HCO_3 in potentiating the conversion of pyruvate to glutamine, but without effect on glutamine synthesis from dipyruvate, glutamate or α-ketoglutarate.

It is reasonable to assume that since oxaloacetate increased the yield of alanine greatly, it was converted in large part to pyruvate. If this is true, then only pyruvate and its conjugate were direct sources of carbon for glutamine synthesis in the experiments where no nitrogen was added. Whatever the reactions involved, some compound or compounds were formed which had a remarkable affinity for nitrogen, greater even than that of pyruvate in alanine synthesis.

Since glutamate and α-ketoglutarate could not serve as precursors of glutamine in the absence of exogenous ammonia, and since components of the tricarboxylic acid cycle (other than oxaloacetate) did not increase liver glutamine, the synthesis of glutamine from pyruvate appears to be an independent pathway. Failure of the tricarboxylic acid cycle compounds to contribute to the synthesis of pyruvate or glutamine could not have been due to permeability factors since both glutamate and α-ketoglutarate were active in the presence of excess ammonia. In addition, glutamate was produced in the liver from α-ketoglutarate, therefore it was available to the cell but the glutamate formed inside the cell was not converted to glutamine (Table 1). It is interesting that malic acid did not provide glutamine either by conversion to α-ketoglutarate in the citric acid cycle or by conversion to pyruvate using the malic enzyme.

Acknowledgement: This work was supported in part by the Edward G. Schlieder Foundation. The authors gratefully acknowledge the technical assistance of Thomas D. Coulson.

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