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Transaminase Activity and Other Enzymatic Reactions Involving Pyruvate and Glutamate in *Chlamydia* (Psittacosis-Trachoma Group)[1]

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ABSTRACT

The agents of meningopneumonitis (MN) and of trachoma (TR) purified from chick embryo allantoic fluids and yolk sacs, respectively, were shown to produce CO_2 from the C_1 positions of pyruvate and glutamate, but not from the other carbon atoms. The reaction with pyruvate did not require the addition of cofactors, but was stimulated to a small extent by α-lipoic acid and, in the case of TR, also by diphosphothiamine, and nicotinamide adenine dinucleotide (NAD). The reaction of MN with glutamate was greatly stimulated by the addition of NAD and pyruvate, and resulted in the accumulation of alanine. The reaction of TR with glutamate was also greatly enhanced by added NAD, but was not affected by added pyruvate. When eight intermediates of the citric acid cycle were added to MN cells incubated with glutamate-C^{14}, plus NAD and pyruvate, they reduced to varying degrees the evolution of $C^{14}O_2$. It was shown by chromatography that the C^{14} label extended to α-ketoglutarate and succinate, but not to fumarate and malate. A net gain in adenosine triphosphate could not be demonstrated in MN cells incubated with combined glutamate, pyruvate, oxaloacetate, and various cofactors. These results furnish additional examples of real or apparent gaps in enzyme sequences in *Chlamydia*.

Previous work from this laboratory (18, 19) has shown that purified suspensions of members of the genus *Chlamydia* [psittacosis-trachoma group (14)] degraded glucose with production of CO_2 and pyruvate. This reaction had cofactor and other requirements that were highly unusual for intact microorganisms. For example, the addition of stoichiometric amounts of adenosine triphosphate (ATP) relative to glucose was required for the liberation of CO_2 from the C_1 position, and the reaction was greatly enhanced by the addition of stoichiometric amounts of nicotinamide adenine dinucleotide phosphate (NADP). The requirement for ATP was eliminated when glucose-6-phosphate was substituted for glucose as the substrate. Moulder et al. (10) demonstrated the presence of glucose-6-phosphate and 6-phosphogluconate dehydrogenases in cell-free extracts of the agent of meningopneumonitis (MN). These enzymes could be differentiated by starch-gel electrophoresis from the corresponding ones derived from uninfected chick embryos. However, Vender and Moulder (*personal communication*) found that hexokinase could be removed from MN particles by digestion with Pronase or trypsin and suggested the possibility that this enzyme was of host origin.

This study was prompted by previous evidence (18, 19) that some of the CO_2 liberated from glucose was derived from the number 3 or 4 carbons, or both, and that pyruvate was possibly an intermediate. In addition to pyruvate, the utilization of glutamate and related compounds was investigated. Most of the work was done with MN purified from allantoic fluid, but a few experiments were carried out with the agent of trachoma (TR) purified from yolk sac. As in previous investigations (18), unusual cofactor effects and apparent gaps in enzyme sequences were encountered.

MATERIALS AND METHODS

The Cal 10 strain of MN was grown in the allantoic fluids of chick embryos, and was purified by the procedure outlined previously (11, 18). The TE-55 strain of TR was grown in yolk sacs, and was purified by a procedure similar to that described by Weiss et al.

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(19). The principal differences between the previous and present procedures were as follows. Pronase (Calbiochem) was substituted for trypsin, and was used during an early as well as a late step in the procedure; the entire procedure of purification was completed during 1 day, instead of 2. The purified suspension was stored at 0 C and was used during the following 2 days in metabolic experiments. Control preparations for MN and TR were prepared as previously described (18, 19) with the modifications introduced here for TR preparations. The final preparations were assayed for protein content and infectivity (19).

Metabolic experiments were carried out with C^{14}-labeled substrates as previously described, except that 25-ml Erlenmeyer flasks were substituted for Warburg vessels. CO_2 was trapped by use of Hyamine (Packard Instrument Co., La Grange, Ill.) placed in plastic cups suspended 1 to 2 cm above the level of the reaction mixtures by plastic rods inserted into the stoppers (3). The flasks, containing in most experiments 2.4-ml volumes of reaction mixtures, were incubated in a metabolic shaker for 2 hr at 34 C plus a 5-min allowance for thermal equilibration. The reaction was stopped by the introduction of 0.6 ml of 25% trichloroacetic acid per flask. After an additional 0.5 hr of incubation, each plastic cup with its contents was separated from its rod and was placed into a liquid scintillation vial. Methanol (2.5 ml) and toluene (15 ml), containing 6 g of 2,5-diphenyloxazole (PPO) per liter and 0.1 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) per liter, were added to each flask, and the radioactivity of the CO_2 was determined in a liquid scintillation counter. The amount of CO_2 produced was calculated as described previously (19) and, unless otherwise indicated, is expressed in millimicromoles per milligram of agent protein. Under these conditions, variation among triplicate tests was usually less than 5%, and the results presented, unless otherwise indicated, are the means of triplicate tests.

In preparation for experiments requiring chromatographic separation of the reaction products, the final volume was 0.8 ml per flask, but the concentrations of microorganisms, 0.4 to 0.5 mg of agent protein, were approximately the same as in other experiments. The reaction was stopped by the addition of 0.2 ml of 2.5 M perchloric acid, and the precipitate was eliminated by centrifugation. To the supernatant fluid was added 0.2 ml of 2.5 M $KHCO_3$, and the precipitated potassium perchlorate was also eliminated by centrifugation. Volumes of 2.5 μliters of these supernatant fluids were chromatographed on plates coated with cellulose, as described in detail by Myers and Huang (12). The contents of selected spots on the cellulose were placed in liquid scintillation vials to which were added toluene containing PPO and POPOP, and Thixotropic gel powder (Cab-O-Sil, Packard Instrument Co.), according to the procedure of Snyder and Stephens (15). The radioactivity of the various spots was presented in terms of disintegrations per minute, based on a counting efficiency of approximately 70%.

RESULTS

Carbon dioxide production from pyruvate and glutamate. MN and TR produced CO_2 from pyruvate and glutamate. Experiments in which pyruvate was labeled with C^{14} in positions 1, 2, and 3 and glutamate was labeled in positions 1, 3–4, and 5 clearly indicated that CO_2 was produced only from carbon 1 of either substrate. Glutamine was also metabolized by both agents, but to an extent somewhat lower than glutamate. Most of the work that followed was carried out with pyruvate-*1*-C^{14} and glutamate randomly labeled with C^{14} (glutamate-*R*-C^{14}).

Typical results are shown in Table 1. MN incubated with pyruvate for 2 hr at 34 C produced, in various experiments, 90 to 140 mμmoles of CO_2 per mg of agent protein. Of three cofactors tested, nicotinamide adenine dinucleotide (NAD), diphosphothiamine, and α-lipoic acid, only α-lipoic acid, added in concentrations of the same magnitude as the substrate, enhanced the reaction by approximately 20%. This small enhancing effect was confirmed in two additional experiments. TR utilized pyruvate less intensely: approximately 28 to 79 mμmoles of CO_2 was produced. The reaction was enhanced to a small extent by added α-lipoic acid and NAD, and to a somewhat greater extent by added diphosphothiamine. When all three cofactors were added (not shown in Table 1), the effect was approximately the same as that of diphosphothiamine alone. The effect of stoichiometric

TABLE 1. *Substrate utilization by the agents of meningopneumonitis and trachoma*

Agent	Substrate (1.7 mM)	Cofactors[a]	CO_2 produced from labeled carbons[b]
MN	Pyruvate-*1*-C^{14}	None	114
		LA[c] (1.7)	136
TR	Pyruvate-*1*-C^{14}	None	79
		LA[c] (2.1)	96
		NAD (4.2)	98
		DPT[c] (4.2)	113
MN	Glutamate-*R*-C^{14}	None	6
		NAD (2.1)	13
TR	Glutamate-*R*-C^{14}	None	20
		NAD (4.2)	127

[a] Numbers in parentheses indicate millimolar concentration.

[b] Millimicromoles per milligram of agent protein per 2-hr incubation period at 34 C; mean of triplicate flasks.

[c] LA, α-lipoic acid; DPT, diphosphothiamine.

amounts of coenzyme A could not be tested, because this cofactor under the conditions of these experiments reacted chemically with pyruvate in the absence of added microorganisms to produce CO_2 from carbon 1.

MN produced a very small amount of CO_2 from glutamate (Table 1). NAD approximately doubled this activity to 9 to 17 mμmoles of CO_2 in various experiments. TR utilized glutamate more intensely, and this activity was very greatly enhanced by NAD (61 to 127 mμmoles).

Evidence of a glutamate-pyruvate transaminase. Table 2 illustrates two attempts to determine the pathway by which glutamate was utilized by MN. The addition of large amounts of unlabeled α-ketoglutarate to glutamate-C^{14} significantly reduced CO_2 production from glutamate. On the other hand, γ-aminobutyrate had no effect. These results can be taken as tentative evidence that glutamate is metabolized via α-ketoglutarate.

The possibility that a transaminase is involved in the catabolism of glutamate was tested by incubating the microorganisms with glutamate-C^{14} and large amounts of unlabeled pyruvate or oxaloacetate. As shown in Table 2, the addition of pyruvate greatly enhanced CO_2 production from glutamate, whereas oxaloacetate was inhibitory. These results suggest that MN possesses a glutamate-pyruvate transaminase.

If MN indeed has an active transaminase, the incubation of MN with pyruvate-C^{14} and unlabeled glutamate should result in the production of C^{14}-labeled alanine. Figure 1 illustrates an experiment designed to test this hypothesis. The microorganisms were incubated with pyruvate-*3*-C^{14} of high specific activity with or without unlabeled glutamate of 10-fold higher concentration. After incubation, the reaction mixtures were treated as described in Materials and Methods and were chromatographed as shown in Fig. 1 with carrier alanine. The radioactivity

TABLE 2. *Glutamate metabolism of MN*

Labeled substrate (1.7 mM)	Unlabeled substrate[a] (17 mM)	CO_2 production from labeled carbons[b]
Glutamate-*R*-C^{14}	None	15
	α-Ketoglutarate	9.5
	γ-Aminobutyrate	15
Glutamate-*1*-3^{14}	None	17
	Pyruvate	62
	Oxaloacetate	10

[a] NAD, 4.2 mM was added to each flask.
[b] See Table 1.

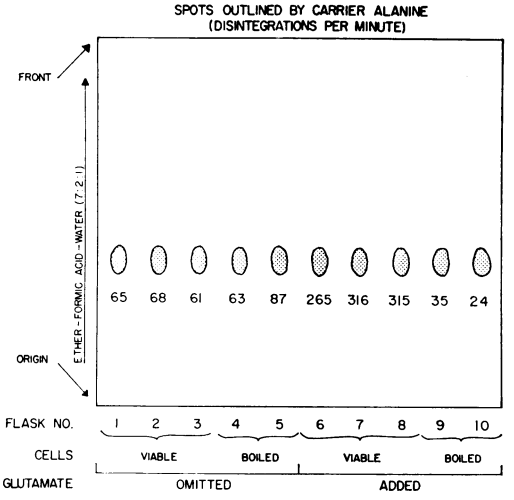

FIG. 1. *Chromatogram of reaction products of MN incubated with pyruvate-3-C^{14} (0.4 mM, 3.12 μc/μmole) without or with 4 mM unlabeled glutamate. Spots were produced with 2.5-μliter samples of reaction mixture plus 2.5 μliters of 0.01 M unlabeled alanine. As a further precaution against the inclusion of host enzymes, the cells used in this experiment, after the routine procedure of purification, were treated for 0.5 hr with Pronase.*

of the alanine spots of the specimens incubated without glutamate was relatively low, and was approximately the same as that of the control preparations composed of boiled microorganisms. The alanine spots of the specimens incubated with glutamate, on the other hand, contained appreciable amounts of radioactivity. The three specimens producing high radioactivity in the alanine spots were processed further by two-dimensional chromatography, which clearly separated alanine from citrate and other intermediates of the citric acid cycle, as outlined in Fig. 2 (broken lines). The radioactivity of the alanine spots remained the same, whereas the spots identified by use of carrier citrate contained no radioactivity. From the volumes used in chromatography, radioactivity obtained in the alanine spots, and specific activity of pyruvate, it can be estimated that under the conditions of these experiments approximately 40 to 50 mμmoles of alanine was produced per mg of protein during the 2-hr period of incubation at 34 C. These amounts are of approximately the same magnitude as the CO_2 produced from glutamate.

The glutamate metabolism of TR did not appear to be affected appreciably by added pyruvate.

Evidence of an α-ketoglutarate dehydrogenase.

The role of the citric acid cycle in the metabolism of MN was further studied in the experiments illustrated in Table 3 and Fig. 2. The microorganisms were incubated with a mixture of glutamate-*R*-C^{14}, NAD, and pyruvate. The effect of each one of eight intermediates was determined and was expressed in terms of the percentage of inhibition of CO_2 production from glutamate. All intermediates interfered to a smaller or larger extent with the CO_2 produced from glutamate-*R*-C^{14}, with the possible exception of citrate and *cis*-aconitate. Fumarate and malate appeared to be the most effective interfering compounds in the first experiment. It appears, however, that the concentration of glutamate and of the other reagents influenced the results. In the second experiment shown in Table 3, carried out primarily as a preparation for chromatography with considerably smaller amounts of glutamate and proportionally higher concentrations of intermediates, α-ketoglutarate appeared to be more effective in reducing CO_2 production from glutamate-*R*-C^{14} than fumarate.

The reaction products of the second experiment were first chromatographed in a single dimension. Since the results obtained in triplicate tests were highly consistent, one specimen in each group was chromatographed in two dimensions (Fig. 2). Surprisingly, the incubation of the microorganisms with labeled glutamate and unlabeled pyruvate, even without the addition of intermediates, led to the accumulation of appreciable amounts of α-ketoglutarate and succinate. The fumarate spot contained virtually no radioactivity at all. The addition of either α-ketoglutarate or fumarate led to a great increase in the accumulation of C^{14} in α-ketoglutarate and a reduction in the accumulation of C^{14} succinate. This reduction was more pronounced with added α-ketoglutarate than with fumarate. However, neither one of these additions resulted in the accumulation of C^{14} in fumarate. In all three cases, the spot identified by carrier malate contained a small amount of radioactivity. The significance of this level of radioactivity or of differences among the three spots is not known.

Succinate and fumarate inhibited CO_2 evolution from glutamate in TR also. The reaction was not investigated further.

Relationship between glucose utilization and other metabolic activities. Since it was clearly shown that ATP is required for the utilization of glucose by MN and TR (18), an attempt was made to use glucose as an indicator of ATP production from some of the other substrates.

TABLE 3. *Effect of citric acid cycle intermediates on glutamate metabolism of MN*

Labeled substrate	Unlabeled substrate[a]	Ratio of labeled to unlabeled substrate	Per centrreduction of $C^{14}O_2$ produced[b]
Glutamate-*R*-C^{14} (2.1 mM)	None		0
	α-Ketoglutarate	1:6.7	36
	Succinate	1:6.7	63
	Fumarate	1:6.7	71
	Malate	1:6.7	72
	Oxaloacetate	1:6.7	59
	Citrate	1:6.7	18
	cis-Aconitate	1:6.7	13
	Isocitrate	1:6.7	37
Glutamate-*R*-C^{14} (0.36 mM)	None		0
	α-Ketoglutarate	1:10	90
	Fumarate	1:10	65

Each flask also contained pyruvate (21 and 3.6 mM, respectively, in the two experiments) and NAD (3.6 and 2.5 mM).

[b] With no unlabeled substrate, the amounts of CO_2 produced per milligram of agent protein per 2 hr were 80 and 30 mμmoles, respectively, with 2.1 and 0.36 mM glutamate-*R*-C^{14}.

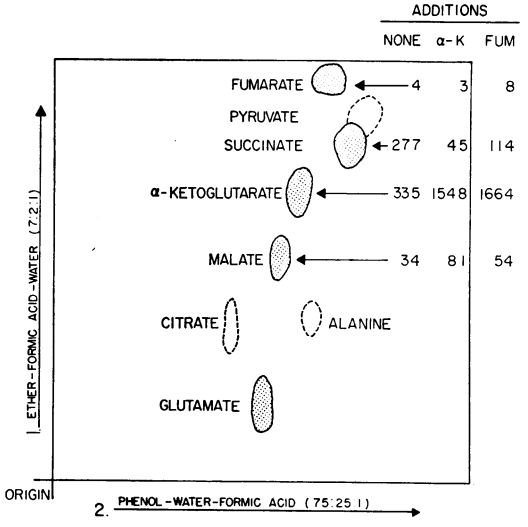

FIG. 2. *Chromatogram of reaction products of MN incubated with glutamate-R-C^{14} (0.36 mM, 10 μc/μmole) without addition or with α-ketoglutarate or fumarate (upper right; see also Table 3). Spots illustrated by cross-hatchings were produced with 2.5-μliter samples of reaction mixture plus 2.5 μliters of a mixture of 0.01 M concentrations of the indicated compounds. Numbers represent disintegrations per minute obtained from spots indicated by arrows under the three experimental conditions. Spots illustrated by broken lines represent two-dimensional chromatograms of specimens shown in Fig. 1. See text.*

As shown in Table 4, added pyruvate reduced CO_2 production from carbon 3 or 4, or both, but not from carbon 1, of glucose-6-phosphate. The reverse was also true. When unlabeled glucose-6-phosphate was added to labeled pyruvate, CO_2 production from pyruvate decreased. This effect was slightly reduced by the simultaneous addition of glucose-6-phosphate and NADP, which enhances CO_2 production from carbon 1 of glucose (18). The addition of unlabeled pyruvate to a mixture of glucose-*1*-C^{14}, NADP, and hexokinase did not result in the accumulation of radioactive CO_2. This result indicates that an appreciable amount of ATP was either not produced or, if produced, did not combine with agent-associated or added hexokinase to produce glucose-6-phosphate.

A more detailed experiment of this nature is shown in Table 5. The unlabeled substrates tested for possible ATP production were pyruvate, glutamate, and oxaloacetate fortified with NAD. Oxaloacetate was included, because preliminary results of E. Weiss and L. A. Kiesow (Bacteriol. Proc., p. 85–86, 1966) indicated that it was also utilized. The test system, as in the previous experiments, consisted of glucose-*1*-C^{14} of high specific activity, NADP, and hexokinase. It is obvious that the addition of the three substrates did not result in utilization of appreciable amounts of glucose. This was also true when the mixture contained adenylic acid (AMP). The addition of adenosine diphosphate (ADP) yielded equivocal results, because, as in previous experiments (18), there was evidence that the agent preparation contained an active adenylate kinase. Relatively large amounts of CO_2 were produced in the absence as well as in the presence of the unlabeled substrates. Under these conditions, a net synthesis of minute amounts of ATP from ADP as the result of substrate utilization would not have been detected. An attempt to eliminate adenylate kinase by treating the purified microorganisms with Pronase was not successful. The results of the last group in Table 5 indicated that without interfering factors, such as adenylate kinase, the test was highly sensitive and probably the formation of amounts as low as 10^{-6} M of glucose-6-phosphate would have been detected.

Metabolic activities of control preparations. Controls for MN consisted of allantoic fluids of uninoculated embryos, to which were added a sufficient number of mechanically disrupted chorioallantoic membranes to raise the protein content to that of infected fluids. Controls for TR consisted of uninfected yolk sacs. Protein yields, according to respective procedures of purification, were compared in each case with those obtained in the three experiments with agents which immediately preceded them. The ratios of protein recovery from control preparations to protein recovery from agent preparations (Table 6) were used to estimate the host protein

TABLE 5. *Effect of substrates and cofactors on glucose metabolism in MN*

Addition[a]	CO_2 from C_1 of glucose[b]	
	Without substrates	With three substrates
None	0.7	0.6
AMP (0.41 mM)	0.5	0.7
ADP (0.41 mM)	92	92
ATP (0.041 mM)	79	67

[a] Each flask contained hexokinase (1 micromolar unit per flask), NAD (4.1 mM), NADP (0.82 mM), and glucose-*1*-C^{14} (0.41 mM).

[b] See Table 1.

[c] Pyruvate, glutamate, oxaloacetate (each 4.1 mM).

TABLE 4. *Interrelationship between glucose and pyruvate metabolisms in MN*

Labeled substrate	Additions	CO_2 production from labeled carbons[a]
Glucose-*3,4*-C^{14} (1.7 mM)	ATP (6.2 mM) hexokinase[b]	81
	ATP, hexokinase, pyruvate (1.7 mM)	30
Pyruvate-*1*-C^{14} (1.9 mM)	None	126
	NADP (1.7 mM)	133
	Glucose-6-phosphate (1.7 mM)	67
	NADP, glucose-6-phosphate	83
Glucose-*1*-C^{14} (1.7 mM)	NADP, hexokinase	0
	NADP, hexokinase, pyruvate (1.7 mM)	0
	NADP, hexokinase, ATP	348
	NADP, hexokinase, pyruvate, ATP	329

[a] See Table 1.

[b] From yeast, highest purity (Sigma Chemical Co., St. Louis, Mo.); approximately 1 micromolar unit per flask.

TABLE 6. *Metabolic activities of control preparations*

Prepn	Protein recovery ratio[a]	Labeled substrate (1.7 mM)	Additions[b]	CO_2 production from labeled carbons[c]
From 160 allantoic fluids 40 CAM	0.10	Pyruvate-*1*-C^{14}	None	0.2
			LA (1.7 mM)	0.0
		Glutamate-*R*-C^{14}	None	1.4
			NAD (4.2 mM)	2.0
From 160 allantoic fluids 40 CAM	0.15	Glutamate-*R*-C^{14}	NAD (4.2 mM)	6.4
			NAD, pyruvate (17 mM)	4.9
			NAD, pyruvate, fumarate (17 mM)	4.9
From 60 yolk sacs	0.067	Pyruvate-*1*-C^{14}	None	0.3
			LA (2.1 mM), NAD, DPT (4.2 mM)	1.2
		Glutamate-*R*-C^{14}	None	3.6
			NAD (3.3 mM)	7.1

[a] Protein recovery from control preparation divided by protein recovery from agent preparation (mean of three previous experiments).
[b] LA, α-lipoic acid; DPT, diphosphothiamine.
[c] Per 1 mg of protein × protein recovery ratio.

contents of agent preparations. These values were probably exaggerated, because in these experiments as well as in previous ones (18) control preparations appeared to have larger numbers of host mitochondria than agent preparations. CO_2 production was corrected on the basis of the fraction of 1.0 mg calculated to represent the host contribution, instead of the usual 1.0 mg of protein.

The contribution of host enzymes to the utilization of pyruvate by either agent preparation appeared to be negligible (Table 6). Since glutamate metabolism of MN with or without NAD was small (Table 1), the low activity of control preparations (Table 6) represented an appreciable fraction of the total activity. However, control and agent preparations differed in two important qualitative aspects. The addition of unlabeled pyruvate tended to reduce glutamate metabolism instead of greatly enhancing it (Table 2). The further addition of fumarate did not reduce the reaction as in case of MN preparations (Table 3). Glutamate was utilized by control yolk sac preparations to a low extent also, but, because of the more intense activity of TR with added NAD, this represented only a small fraction of the total.

On the basis of these results, it can be concluded that the host contributed some glutamate dehydrogenase to the reactions illustrated in Tables 1 to 3, but only negligible amounts of other enzymes.

DISCUSSION

Pyruvate and glutamate are utilized by both MN and TR. The reaction with pyruvate requires no added cofactors, although some exercise a moderately stimulatory effect. The reaction with glutamate is almost entirely dependent on added NAD and, in the case of MN, on pyruvate, which serves as an acceptor of the amino group. The role of phosphorylated compounds in the metabolism of intact chlamydial cells was discussed in a previous publication (18). The stimulation by α-lipoic acid of the decarboxylation of pyruvate resembles the phenomenon described by Gunsalus, Dolin, and Struglia (5) in *Streptococcus faecalis*.

The reaction of both MN and TR with pyruvate can best be interpreted as oxidative decarboxylation. These experiments have provided no evidence that the resulting 2-carbon fragments are further catabolized.

The most active enzyme of MN reacting with glutamate appears to be a glutamate-pyruvate transaminase. The reaction that takes place without added pyruvate can be attributed to a dehydrogenase, but other possibilities should be considered. It can be due to the transaminase acting in concert with endogenously produced pyruvate, or to a decarboxylase. The results also indicate the presence of an α-ketoglutarate dehydrogenase.

The reaction of TR with glutamate is almost entirely dependent on added NAD and can best be interpreted as being due to glutamate and α-ketoglutarate dehydrogenases. The presence of smaller amounts of transaminase or decarboxylase cannot be ruled out.

Gordon and Quan (4) have divided chlamydiae into subgroups A and B on the basis of the degree of compactness or diffusion of the cyto-

plasmic inclusion and the presence or absence of glycogen. Lin and Moulder (8) have added the criteria of sensitivity or resistance to sulfadiazine and to D-cycloserine to the differentiation of these subgroups. TR and MN are typical members of subgroups A and B, respectively. The difference in transaminase activity described here may be due entirely to the physiological conditions of the two microorganisms, because MN and TR were grown in different tissues of the chick embryos. However, if the nature of the difference is genetic, it might be worthwhile to explore whether it can be used as an additional criterion to separate subgroups A and B.

The evidence presented in Fig. 2 indicates that MN cells entered the citric acid cycle with the production of α-ketoglutarate and succinate from glutamate. The reaction stopped there, and fumarate and malate were not formed. Kiesow and Weiss (*unpublished data*) showed by dual wavelength spectrophotometry that, in addition to α-ketoglutarate, *cis*-aconitate and, possibly, oxaloacetate reduced endogenous pyridine nucleotides. No reduction of endogenous or added NAD or NADP was obtained with the other intermediates.

These results contrast with those presented in Table 3, which indicate that, in addition to α-ketoglutarate, most, if not all, intermediates of the citric acid cycle reduced CO_2 production from glutamate. A satisfactory explanation cannot be offered for the effect of the intermediates other than α-ketoglutarate, except that Huang (*unpublished data*) has recently shown that, in *Rickettsia quintana*, *cis*-aconitate interferes with the uptake of glutamine.

Of the various explanations that can be offered for the apparent gap in a natural sequence of enzymes in cells that have not been obviously damaged or specifically inhibited, the following three merit consideration. (i) The function of above-described reactions is just to synthesize a few intermediates for synthesis. (ii) The cells contain all the enzymes of the citric acid cycle, but the specific coenzyme must be added in each case, even though the reactions are carried out by whole cells. (iii) The cells contain other enzymes, but, in the particular stage of development in which they are studied, the synthesis of these enzymes has been repressed. MN cells are harvested after they have disrupted their host cells and are free in the allantoic fluid (11). Under these conditions, most of the cells are of the "small-particle" type. The possibility should be entertained that, during an early stage of intracellular growth when most cells are of the "large-particle" type (9), additional enzymes are present.

There is no evidence that the reactions thus far described result in the production of useful energy. This is not surprising, because Weiss and Kiesow (Bacteriol. Proc., p. 85–86, 1966) showed that MN cells have no demonstrable flavoproteins or cytochromes and that they can reduce pyridine nucleotides but cannot reoxidize them. Thus, even if MN has all the enzymes of the citric acid cycle, for proper function the host cell must provide cofactors and reoxidize reduced cofactors.

From the vantage point of these new observations, the relationship of chlamydiae to other microorganisms can be re-examined. The utilization of glutamate, in preference to glutamine, is a property that chlamydiae share with the typhus rickettsiae and *R. rickettsii* (2, 6; E. Weiss, H. B. Rees, Jr., and J. R. Hayes, Nature, *in press*). On the other hand, *R. quintana*, which grows on blood-agar (17), utilizes glutamine in preference to glutamate (K. Huang and E. Weiss, Bacteriol. Proc., p. 116–117, 1965). The same is true of *Wolbachia persica* (16), which is an obligate intracellular parasite, but has a very active independent metabolism (13, 20). Glutamate may well be an important metabolite for a number of obligate intracellular parasites, but similarities between the metabolisms of chlamydiae and rickettsiae are only superficial. Typhus rickettsiae (1) as well as *R. quintana* have a glutamate-oxaloacetate transaminase and produce CO_2 without added cofactors from all carbons of glutamate (7; K. Huang, *unpublished data*). Thus, chlamydiae still retain a unique position among the obligate intracellular bacteria that have been studied thus far.

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