Role of Exogenous Adenosine Triphosphate in Catabolic and Synthetic Activities of *Chlamydia psittaci*

EMILIO WEISS AND NORALEE N. WILSON

Department of Microbiology, Naval Medical Research Institute Bethesda, Maryland 20014

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The synthetic activities of isolated cells of the meningopneumonitis strain (MN) of *Chlamydia psittaci* were investigated and further observations were made on their catabolic reactions. These observations included the demonstration of CO₂ production from aspartate in the presence of pyruvate and the formation of pyruvate from glucose-6-phosphate. Both reactions were enhanced by added adenosine triphosphate (ATP). Of a large number of compounds tested, only glucose-6-phosphate, pyruvate, aspartate, and isoleucine were shown to furnish carbons that were incorporated into molecules precipitated by trichloroacetic acid. The reactions with pyruvate, aspartate, and isoleucine were dependent entirely, or almost entirely, on added ATP, and the reaction with glucose-6-phosphate was enhanced by ATP. Except for CO₂, which greatly stimulated the reactions, the addition of a number of other compounds or a combination of compounds, such as cofactors, amino acids, and purine and pyrimidine bases, did not greatly affect incorporation. About 95% of the activity of the trichloroacetic acid precipitates was recovered in the chloroform-methanol soluble fraction.

It has been shown that isolated cells of the host-dependent genus *Chlamydia* can carry out a number of catabolic reactions (9-13), but these reactions have cofactor and other requirements that are unusual for intact microorganisms and it is unlikely that they yield useful energy. One of these reactions, catabolism of glucose, is dependent on the addition of stoichiometric amounts of adenosine triphosphate (ATP) relative to glucose (9). Vender and Moulder (8) found, however, that hexokinase could be removed from some chlamydial cells by digestion with Pronase or trypsin and suggested the possibility that this enzyme was of host origin and that ATP acted on this enzyme and was not a true requirement of the chlamydial cell.

This study had the dual purpose of determining whether chlamydial cells, in addition to degrading substrates, incorporated them into large molecules and whether these reactions required ATP. The microorganism used for this study was the meningopneumonitis (MN) strain of *C. psittaci*. Both questions were answered in the affirmative.

MATERIALS AND METHODS

The Cal-10 strain of MN was grown in the allantoic fluid of chick embryos, harvested, and purified as

previously described (9, 13). Substrates labeled with ¹⁴C, cofactors, enzymes, and compounds of intermediary metabolism were obtained from New England Nuclear Corp. (Boston, Mass.) and Calbiochem (Los Angeles, Calif.).

Procedures for the determination of CO₂ production, collection of the reaction products, and thin-layer chromatography were described previously (10). ¹⁴C-pyruvate was isolated with the help of unlabeled pyruvate by one- or two-dimensional development with ether, formic acid, and water in a ratio of 7:2:1 (7). Pyruvate-*l*-¹⁴C was estimated by treating the reaction products with H₂O₂ and trapping the CO₂. ¹⁴C₁ of pyruvate was quantitatively released as ¹⁴CO₂ under these conditions, whereas ¹⁴C₂ and ¹⁴C₃ were not so affected. ¹⁴CO₂ obtained from comparable specimens treated with distilled water, instead of H₂O₂, was subtracted.

Incorporation experiments were carried out in screw-cap tubes (100 by 16 mm). To each tube, we added 0.4 ml of a suspension of microorganisms suspended in diluent K 36 (9) plus 2.5 mg of bovine plasma albumin and 0.8 ml of a mixture of the needed reagents, including the ¹⁴C-labeled substrate. The tubes were flushed for 10 sec with a mixture of 5% CO₂ in air, tightly stoppered, and incubated at 34°C in a water bath with gentle shaking. A typical experiment consisted of four groups of four tubes. Two tubes of each group were processed at 2 hr and the

rest at 4 hr. At these time intervals, the tubes were placed in ice water for 5 min and were centrifuged for 45 min at $3,000 \times g$. The supernatant liquid was removed and each pellet was suspended in 2 ml of K 36 containing a 1 to 10 mM solution of unlabeled substrate corresponding to the labeled substrate being tested. To each tube, we added 2 ml of 10% trichloroacetic acid. The specimens were maintained in ice water for 0.5 hr; the total volumes or 1- to 2-ml portions were passed through Millipore filters (diameter 25 mm, pore size 0.3 μ m), and the filters were washed five times with 5% trichloroacetic acid. Some of the filters were then placed in 20-ml vials, 12 ml of Bray's solution (4) was added to each vial, and the radioactivity was determined in a liquid scintillation counter. Other filters were treated twice for 15-min periods with 3 ml of a 2:1 mixture of chloroform and methanol. The chloroform-methanol washes were collected and dried at 56 C under nitrogen. The radioactivity of the treated filters and the eluates was determined as above. When microorganisms were mixed with a radioactive compound and immediately processed, the filters, before chloroform-methanol treatment, retained approximately 20 disintegrations per minute (dpm) for each 1,000,000 dpm added, but nonspecific retention was probably higher after incubation. In most incorporation experiments, variation between duplicates was less than 10%, and differences between groups of greater than 10%, obtained both at 2 and 4 hr, were highly reproducible.

All results are presented as the means of duplicate or triplicate specimens in terms of nanomoles (per milligram of cell protein or per flask) of CO_2 , carbon, or pyruvate, calculated from the radioactivity of the specimens examined and the specific activity of the substrates before the reaction.

RESULTS

Of a large number of compounds tested, isolated MN cells were shown to incorporate, into fractions precipitated by trichloroacetic acid, significant amounts of carbon of only four substrates: glucose-6-phosphate, pyruvate, aspartate, and isoleucine. As reported in previous publications (5, 9-13) and expanded below, CO_2 and compounds of intermediary metabolism were produced from the first three compounds but not from isoleucine.

Catabolic activities. MN cells incubated with labeled aspartate produced low levels of CO_2 (Table 1). The reaction required added pyruvate and was enhanced by added ATP. When the reciprocal experiment was performed with pyruvate labeled in carbon position 1, added aspartate did not affect the reaction and ATP, if at all, slightly depressed it.

It was previously shown that glucose-6-phosphate is catabolized via the initial steps of the pentose pathway to produce CO_2 from C_1 and via another pathway, most likely glycolytic, to

TABLE 1. Carbon dioxide production from aspartate and pyruvate^a

Expt no.	Labeled substrate	Addition	CO_2 formed from labeled carbon ^b
1	¹⁴ C-Aspartate (randomly labeled; 1.7 mm, 0.05 μ c/ μ mole)	None	2.6
		ATP (4.2 mm)	8.8
		Pyruvate (1.7 mm)	21.9
		ATP, pyruvate	34.2
2	Pyruvate-1- ¹⁴ C (1.7 mm, 0.1 μ c/ μ mole)	None	92.4
		ATP (4.2 mm)	84.0
		Aspartate (4.2 mm)	93.6
		ATP, aspartate	82.5

^a Each flask contained 7.5 mg of bovine plasma albumin (Pentex), 1.7 mm MgCl_2 , and 0.34 mm MnCl_2 . The MN cell protein content in the two experiments was 0.58 and 0.33 mg/flask. The total volume was 2.4 ml.

^b Nanomoles per milligram of agent protein after 2 hr of incubation at 34 C. Mean of triplicate flasks.

form pyruvate (12). Most of these experiments were carried out with glucose, hexokinase, and an excess of ATP. It is not known, therefore, whether ATP was actually required for the production of pyruvate from glucose-6-phosphate. An attempt to clarify this point is illustrated in Table 2. MN cells were incubated with low concentrations of glucose-6-phosphate, labeled to a high degree of specific activity in the C_1 position or labeled randomly, and with very large concentrations of unlabeled pyruvate, to prevent rapid degradation of pyruvate derived from glucose-6-phosphate. Pyruvate was isolated by thin-layer chromatography, and the amount derived from glucose-6-phosphate was estimated from its radioactivity. It is clear that pyruvate was produced from glucose-6-phosphate in the presence or absence of exogenous ATP, but its production was approximately doubled when exogenous ATP was present. Equivalent amounts of labeled pyruvate were isolated from C_1 and randomly labeled glucose-6-phosphate. The carboxylic group of pyruvate was labeled only when randomly labeled glucose-6-phosphate was used.

Synthetic activities. A study of the incorporation of glucose is illustrated in Table 3. Glucose-6-¹⁴C was selected because it was previously shown (12) that CO_2 is not produced from C_6 . Some radioactivity was precipitated by trichloroacetic acid even when the cells were incubated without ATP, but this amount, approximately

TABLE 2. *Metabolism of labeled carbon from glucose-6-phosphate^a*

Glucose-6-phosphate		Addition (nmoles per flask)		Labeled reaction products ^b (nmoles per flask)		
Position of labeled carbon	Amt added (nmoles per flask)	Pyruvate	ATP	CO ₂	Pyruvate (minimal values)	C ₁ -labeled pyruvate (maximal values)
1	140	8,000		54	6.0	
1	140	8,000	4,000	50	10.5	0.0
1-6	70	8,000		51	3.5	
1-6	70	8,000	4,000	49	6.6	5.4

^a Each flask contained 1.1 mg of cell protein in a total volume of 0.8 ml. The concentrations of bovine plasma albumin and of divalent salts were the same as those of the experiments described in Table 1. The substrates were glucose-1-¹⁴C-6-phosphate, 10 μ C/ μ mole, or randomly labeled glucose-6-phosphate, 35 μ C/ μ mole. The flasks were incubated at 34 C for 2 hr.

^b Since pyruvate was isolated by thin-layer chromatography and quantitative recovery was not expected, the data presented are considered "minimal values." C₁-labeled pyruvate was estimated from ¹⁴CO₂ released by H₂O₂ from samples of the specimens before chromatography. Since the specimens may have contained trace amounts of other keto acids contributing ¹⁴CO₂, these data are considered "maximal values."

0.10 to 0.15 nmoles of carbon per tube, is not believed to represent specific incorporation. When glucose was converted to glucose-6-phosphate by the addition of ATP and hexokinase and the tubes were flushed with CO₂, a small but highly significant amount of C₆ was incorporated. In subsequent experiments, all tubes were flushed with CO₂.

Although in most experiments the amount of carbon incorporated at 4 hr was approximately double that obtained at 2 hr, a pronounced increase in incorporation was not obtained when incubation was extended beyond 4 hr. The incorporation of glucose-6-phosphate was not stimulated by the addition of various cofactors, such as nicotinamide adenine dinucleotide phosphate (NADP) or various combinations of amino acids, purine and pyrimidine bases, and other compounds. In contrast, NADP greatly stimulated CO₂ production from glucose-6-phosphate (9, 13).

As shown in Table 4, pyruvate and aspartate, which are themselves incorporated, when added to labeled glucose-6-phosphate, slightly depressed its incorporation. This effect can be interpreted as a radioisotope dilution effect of a common intermediate, since total carbon incorporation was

increased. This increase was demonstrated in an experiment, not shown in Table 4, in which each compound, in turn, was labeled.

When glucose-6-phosphate was used, instead of a mixture of glucose, ATP, and hexokinase, this substrate was incorporated without added ATP, but added ATP was stimulatory (Table 4). Excess pyruvate reduced glucose-6-phosphate incorporation only when it was stimulated by ATP. Two potential intermediates, ribose-5-phosphate and phosphoenolpyruvate, were also tested for their diluting effect of the specific activity of glucose-6-phosphate. This was apparently accomplished only with the relatively large concentrations shown in Table 4.

The results in Tables 3 and 4 are presented in terms of labeled carbon rather than in terms of glucose incorporated. The ratio between incorporation, under comparable conditions, of C₆ and all carbons of glucose-6-phosphate was approximately 1:3.

Although no added factor is required for the release of CO₂ from C₁ of pyruvate, incorporation of C₃ was found to be strictly dependent on added ATP (Table 5). Aspartate did not affect the reaction. As in the case of glucose-6-phosphate, the addition of large concentrations of phosphoenolpyruvate reduced incorporation of pyruvate. Acetate was incorporated to a considerably smaller degree than pyruvate.

Synthetic reactions involving aspartate and isoleucine are illustrated in Tables 6 and 7. The incorporation of both compounds was dependent, to a large extent, but not entirely, on added ATP. Evidence that a very small portion of the reaction did not require ATP was obtained in an

TABLE 3. *Incorporation of carbon from glucose^a*

Addition	C ₆ incorporated ^b	
	2 hr	4 hr
None.....	0.26	0.33
CO ₂ ^c	0.17	0.30
ATP (5 mM) hexokinase ^d	1.65	3.5
CO ₂ , ATP, hexokinase.....	5.2	11.3

^a The substrate was glucose-6-¹⁴C, 1 mM, 0.5 μ C/ μ mole. Each tube also contained 2.5 mg of bovine plasma albumin, 1.2 mM MgCl₂, and 0.24 mM MnCl₂. MN cell protein content: 0.53 mg/flask. The total volume was 1.2 ml.

^b Nanomoles per milligram of agent protein; mean of duplicate tubes. The tubes were incubated at 34 C.

^c Each tube was flushed for 10 sec with a mixture of 5% CO₂ in air. In subsequent experiments, all tubes were so treated.

^d One-half to one micromolar unit.

TABLE 4. *Effect of various compounds on the incorporation of carbon from glucose-6-phosphate*

Expt no.	Labeled substrate ^a	Addition	Labeled carbon incorporated ^b	
			2 hr	4 hr
1	Glucose-6- ¹⁴ C (1 mM)	None	0.26	0.33
		ATP (5 mM), hexokinase	6.8	11.4
		ATP, hexokinase, pyruvate (1 mM)	5.6	9.4
		ATP, hexokinase, pyruvate, aspartate (1 mM)	4.7	8.4
2	Glucose-6-phosphate- ¹⁴ C (randomly labeled, 1 mM)	None	20.6	32.0
		Pyruvate (10 mM)	19.8	31.2
		ATP (5 mM)	28.8	40.2
		ATP, pyruvate	22.8	33.8
3	Glucose-6-phosphate- ¹⁴ C (randomly labeled, 0.5 mM)	None	19.8	29.8
		Ribose-5-phosphate (10 mM)	15.4	21.3
		Phosphoenolpyruvate (10 mM)	12.9	21.1

^a The specific activity of the substrate varied from 0.3 to 0.6 $\mu\text{C}/\mu\text{mole}$.

^b Nanomoles of carbon per milligram of agent protein. Protein content in the three experiments was 0.53, 0.61, and 0.48 mg per tube.

TABLE 5. *Incorporation of carbon from pyruvate and acetate*

Expt no.	Labeled substrate ^a	Addition	Labeled carbon incorporated ^b	
			2 hr	4 hr
1	Pyruvate-3- ¹⁴ C (1 mM)	None	0.16	
		ATP (5 mM)	3.8	
		Aspartate (2.5 mM)	0.14	
		ATP, aspartate	3.4	
2	Pyruvate-3- ¹⁴ C (1 mM)	None	0.15	0.22
		ATP (5 mM), aspartate (2.5 mM)	4.5	8.4
3	Pyruvate-3- ¹⁴ C (1 mM)	None	0.53	0.57
		ATP (5 mM)	3.4	5.5
		ATP, phosphoenolpyruvate (10 mM)	1.8	2.3
4	Acetate-1,2- ¹⁴ C (1 mM)	None	0.54	0.58
		ATP (5 mM)	2.50	2.65
		ATP, pyruvate (1 mM)	2.50	2.55

^a The specific activity of the substrate was approximately 0.5 $\mu\text{C}/\mu\text{mole}$.

^b Nanomoles of carbon per milligram of agent protein. Protein content in the four experiments was 0.58, 0.47, 0.58, and 0.25 mg/tube.

experiment in which the radioactivity was maintained at a constant level, but the specific activity of the substrates was changed by the addition of different amounts of the unlabeled compounds. Under these conditions, the radioactive fraction precipitated by trichloroacetic acid increased with increasing specific activity (Table 7). In a similar experiment conducted with glucose-6-¹⁴C and pyruvate-3-¹⁴C, the addition of un-

labeled substrates did not influence the amount of radioactivity retained by the filters.

In contrast to catabolic reactions, aspartate incorporation was not affected by added pyruvate, but was reduced somewhat by glucose-6-phosphate. Isoleucine incorporation was stimulated to a very slight degree, if at all, by pyruvate and glucose-6-phosphate (Table 6).

Surprisingly, aspartate and isoleucine ap-

TABLE 6. *Incorporation of carbon from aspartate and isoleucine*

Expt no.	Labeled substrate	Addition	Labeled carbon incorporated ^a	
			2 hr	4 hr
1	¹⁴ C-Aspartate (randomly labeled; 1 mM, 1 μ C/ μ mole)	None	3.3	3.3
		ATP (5 mM)	13.0	17.6
		ATP, pyruvate (1 mM)	13.2	18.5
		ATP, pyruvate, glucose-6-phosphate (1 mM)	8.8	16.7
2	¹⁴ C-Isoleucine (randomly labeled; 1 mM, 1 μ C/ μ mole)	None	0.9	1.1
		ATP (5 mM)	9.2	12.0
		ATP, pyruvate (1 mM)	10.2	13.5
		ATP, pyruvate, glucose-6-phosphate (1 mM)	11.9	13.1

^a Nanomoles of carbon per milligram of agent protein. Protein content in the two experiments was 0.46 and 0.47 mg/ml.

peared to be the only two amino acids of those tested to be incorporated to an appreciable extent by MN cells. Using small concentrations of amino acids labeled to a high degree of specific activity, slight incorporation of leucine and valine could be demonstrated, but such incorporation of 11 other amino acids could not (Table 8). Among these amino acids was glutamate, which is readily catabolized to succinate in the presence of pyruvate (10).

The trichloroacetic acid precipitates obtained from several experiments illustrated in Tables 4 to 7 were further fractionated. In almost every case, approximately 95% of the radioactivity was recovered in the fraction soluble in methanol-

TABLE 7. *Incorporation of carbon from various concentrations of aspartate and isoleucine*

Labeled compound ^a	Molarity (concn, mM)	Labeled carbon incorporated ^b			
		2 hr		4 hr	
		No addition	ATP added	No addition	ATP added
Aspartate	1.003		597		974
	0.103	207	4,402	296	6,628
	0.013	638	23,764	745	29,636
Isoleucine	1.002		272		420
	0.102	501	2,203	1,021	2,992
	0.012	2,475	11,434	4,502	15,422

^a Each tube contained approximately 0.6 μ C of either aspartate or isoleucine randomly labeled with ¹⁴C.

^b Expressed as dpm per tube. With the highest concentrations of substrate, the results were approximately the same as those shown in Table 6.

TABLE 8. *Incorporation of carbon from amino acids^a*

Carbon incorporated ^b	Amino acids
2.0-1.0	Aspartate (1.86), ^c isoleucine (1.72)
1.0-0.2	Leucine (0.47), valine (0.27)
0.2-0.1	Serine, histidine, glutamic acid, tyrosine, phenolalanine, arginine
0.1-0.01	Lysine, proline, threonine, alanine, glycine

^a After 2 hr at 34 C. Each tube contained MN cell (0.36 mg of protein), 5 mM ATP, 1 mM pyruvate, and a 0.008 to 0.012 mM concentration of the amino acid to be tested, randomly labeled with ¹⁴C, 10 μ C/ μ mole.

^b Nanomoles of carbon per milligram of agent protein. The number of carbon atoms of each amino acid was taken into consideration.

^c Amount of carbon incorporated is given for the first four amino acids listed. The rest of the values are of doubtful significance.

chloroform, suggesting that the activity studied in these experiments was lipid synthesis. E. M. Neptune, Jr., R. W. Gaugler, and G. M. Adams (Federation Proc. p. 332, 1968) further analyzed comparable reaction mixtures and recovered the radioactivity in the fatty acids of the phospholipids, principally phosphatidyl ethanolamine. In subsequent studies (*in preparation*), these authors showed that, unlike the chick embryo host cells, MN synthesized a large proportion of branched-chain fatty acids.

DISCUSSION

This work was in part prompted by the results of Bovarnick (1), Bovarnick and Schneider (2),

and Bovarnick, Schneider, and Walter (3), who demonstrated that isolated typhus rickettsiae are capable of synthesizing small amounts of protein and minute amounts of lipid from exogenous substrates. There was no evidence that MN synthesized protein in our experiments, even when provided with all of the reagents required by rickettsiae. The extent of lipid synthesis by MN appeared to be of the same order as protein synthesis by rickettsiae and at least 10 times greater than their lipid synthesis. The limited incorporation of acetate- $1\text{-}^{14}\text{C}$ into lipid by typhus rickettsiae was interpreted by Bovarnick (1) as being due to a radioisotope dilution effect. Rickettsiae presumably produced most of the needed acetyl-coenzyme A from unlabeled glutamine, which was needed for energy. A similar dilution effect did not occur in our experiments with MN.

Several examples of reactions requiring exogenous ATP have been described, and the existence of this requirement in MN can now be regarded as established. In some cases, limited energy may have been provided by compounds such as glucose-6-phosphate or pyruvate (Tables 1, 2, and 4). Rickettsiae, too, require exogenously supplied ATP for their synthetic activities, but exogenous ATP is not enough and an energy-yielding substrate, such as glutamate or glutamine, must also be provided (2). There was no evidence that MN has such a dual requirement.

MN appeared to be less dependent than rickettsiae on added factors for lipid synthesis (1). It is not known whether this is a true physiological difference or whether it merely reflects the extent of cell damage, since both microorganisms must be subjected to arduous procedures of purification before they are used as isolated cells.

The experiment illustrated in Table 2 confirms previous indications (12) that MN follows separate pathways for the production of pentose phosphates and pyruvate from glucose-6-phosphate. The glycolytic pathway is the most likely one to be involved in the production of pyruvate. Phosphoenolpyruvate is a likely intermediate for lipid synthesis from both glucose-6-phosphate and pyruvate. If this is true, added ATP appears to be essential for phosphoenolpyruvate production from pyruvate, but not from glucose-6-phosphate (Tables 4 and 5).

It is plausible that aspartate is utilized in part for the production of diaminopimelate and lysine, since both pyruvate and ATP are needed for optimal CO_2 production (Table 1). Synthesis of lysine from diaminopimelate was demonstrated by Moulder, Novosel, and Tribby (6). The

pathway leading to lipid formation is most likely different, because the addition of pyruvate is not required for this reaction (Table 6) and lysine is not incorporated (Table 8).

The significance of our finding, that of all the compounds tested only glucose-6-phosphate, pyruvate, and two amino acids were incorporated and only lipid was formed, remains unknown. A successful parasite retains the ability to produce compounds that cannot be pilfered from the host cell. The recovery of radioactivity from branched-chain fatty acids of phosphatidyl ethanolamine (Neptune et al., Federation Proc., p. 332, 1968; *in preparation*) seems to indicate that the activities of MN described here fall into this category.

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