UNCLASSIFIED
SECURITY CLASSIFICATION OF THIS PAGE

(2)

REPORT DOCUMENTATION PAGE

AD-A207 948

1b. RESTRICTIVE MARKINGS

3. DISTRIBUTION/AVAILABILITY OF REPORT
Approved for public release; distribution is unlimited

2b. DECLASSIFICATION/DOWNGRADING SCHEDULE

4. PERFORMING ORGANIZATION REPORT NUMBER(S)
NMRI 89-15

5. MONITORING ORGANIZATION REPORT NUMBER(S)

6a. NAME OF PERFORMING ORGANIZATION
Naval Medical Research

6b. OFFICE SYMBOL (If applicable)

7a. NAME OF MONITORING ORGANIZATION
Naval Medical Command

6c. ADDRESS (City, State, and ZIP Code)
Bethesda, Maryland 20814-5055

7b. ADDRESS (City, State, and ZIP Code)
Department of the Navy
Washington, D.C. 20372-5120

8a. NAME OF FUNDING/SPONSORING ORGANIZATION Naval Medical Research and Development Command

8b. OFFICE SYMBOL (If applicable)

9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER

8c. ADDRESS (City, State, and ZIP Code)
Bethesda, Maryland 20814-5055

10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
61102A	3M161102BS13	AK111	DA313955

11. TITLE (Include Security Classification)
Energy metabolism of monocytic Ehrlichia

12. PERSONAL AUTHOR(S)
Weiss E, Williams JC, Dasch GA, Kang Y

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
journal article	FROM _____ TO _____	1989	5

16. SUPPLEMENTARY NOTATION
Reprinted from: Proceedings of the National Academy of Sciences Vol.86, March 1989 pp.1674-1678

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Intracellular bacteria Animal pathogens Human pathogens

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS

21. ABSTRACT SECURITY CLASSIFICATION
Unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL
Phyllis Blum, Information Services Division

22b. TELEPHONE (Include Area Code)
202-295-2188

22c. OFFICE SYMBOL
ISD/ADMIN/NMRI

Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 1674–1678, March 1989
Microbiology

Energy metabolism of monocytic *Ehrlichia*

(intracellular bacteria/animal pathogens/human pathogens)

EMILIO WEISS*†, JIM C. WILLIAMS‡§, GREGORY A. DASCH*, AND YUAN-HSU KANG*

*Naval Medical Research Institute, Bethesda, MD 20814; ‡U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD 21201; and §Office of the Director of Intramural Research Program, National Institute of Allergy and Infectious Diseases, Bethesda, MD 20892

Communicated by Carl R. Woese, December 1, 1988

ABSTRACT **We investigated if the monocytic *Ehrlichia* are totally dependent on their host cells for energy, or, as *Rickettsia*, are capable of some ATP synthesis *in vitro*. The Miyayama strain of *Ehrlichia sennetsu* and the Maryland and Illinois strains of *Ehrlichia risticii* were cultivated in a mouse macrophage cell line, separated from host cell constituents by procedures that included Renografin or Percoll gradient centrifugation, and tested after cryopreservation. Cells incubated without a metabolizing substrate contained little, if any, ATP. When the *Ehrlichia* cells were incubated for 1 hr at 34°C with glutamine, significant amounts of ATP were detected. The amounts of ATP attained with glutamine were decreased in some instances by the addition of atractyloside, an inhibitor of adenine nucleotide translocase in mitochondria, and were decreased consistently and to a greater extent by 2,4-dinitrophenol. When ATP, instead of glutamine, was added to the ehrlichiae, upon incubation the amount of ATP was markedly decreased. Comparable responses under all these conditions were obtained with *Rickettsia typhi*, although the final ATP levels were higher. Control preparations derived from uninfected mouse macrophages or from the discards of the *Ehrlichia* purification procedures contained negligible amounts of ATP, which were not increased by incubation with glutamine. We conclude that with respect to ATP metabolism, the monocytic *Ehrlichia* resemble *Rickettsia* more closely than *Chlamydia*, even though *Ehrlichia* resemble *Chlamydia* in their intracellular location in the phagosomes and in possibly having a developmental cycle.**

The three species of *Ehrlichia* that grow preferentially in the phagosomes of monocytic cells, *E. canis*, *E. sennetsu*, and *E. risticii*, the latter two species in particular, greatly resemble each other in morphological and developmental characteristics and antigenic composition (1). This similarity contrasts with their differences in host affinity and geographic distribution. *E. sennetsu*, isolated in 1953 in Japan (2), is a human pathogen believed to be confined to the Far East (3), while *E. risticii*, isolated in 1985 (4, 5), is associated with a disease of horses, observed in the eastern United States about a decade ago. This contrast renders the understanding of the natural history and phylogeny of these microorganisms quite difficult. Several approaches are required to reach such an understanding, including an investigation of the biochemical characteristics of *Ehrlichia*.

In previous studies (6, 7) we have shown that *E. sennetsu* and *E. risticii*, separated from host cell constituents, utilize glutamine in preference to other substrates that have been tested, including glutamate. There is no indication that they have a glycolytic pathway. In this respect they resemble the obligate intracellular rickettsiae, although these microorganisms utilize glutamate somewhat better than glutamine (8). In the study here reported we show that *Ehrlichia* derive some ATP from the metabolism of glutamine, as is the case in rickettsiae (8, 9).

MATERIALS AND METHODS

Bacterial Strains. The isolation histories of the Miyayama strain of *E. sennetsu* and the Maryland and Illinois strains of *E. risticii* and their cultivation in the $P388D_1$ mouse macrophage cell line were described in a previous publication (7). The Wilmington strain of *Rickettsia typhi* was cultivated and harvested from the yolk sacs of chicken embryos, as described (9).

Separation of *Ehrlichia* and *Rickettsia* from Host Constituents. For the early experiments, the separation of the ehrlichiae from host constituents was done by the Renografin gradient centrifugation procedure without prior proteolytic digestion, as previously described (7). The diluent used in this procedure consisted of 0.2 M sucrose and 0.05 M potassium phosphate buffer, pH 7.4 (SPK). For the final step in the procedure, for cryopreservation, and for the metabolic tests, $MgSO_4$ was added to a final concentration of 5 mM. For the more recent tests, the preparations of the heavily infected cells, after mechanical disruption, were subjected to three 20-min steps of enzymatic digestion at room temperature. First, trypsin was added to a final concentration of 500 μg/ml, then DNase in two steps to a final concentration of 250 μg/ml. The DNase contained sufficient $MgSO_4$ to bring the final magnesium concentration to 5 mM. The DNase added in the final step also contained Bowman–Birk trypsin–chymotrypsin inhibitor (Sigma) to a final concentration of 125 μg/ml. After digestion, the preparations were further purified by filtration through type AP-20 glass microfiber depth filter (Millipore) and concentrated by centrifugation. The ehrlichiae were separated from host constituents by isopycnic centrifugation in 25 ml of 32% Percoll gradients (Pharmacia) using a Ti 70 rotor (Beckman) for 30 min at 63,000 × g_{max}. To the SPK diluent were added glutamine, final concentration 1 mM, and for some of the steps in the procedure 1 mM citric acid. *R. typhi* was purified by the Renografin procedure as described (9).

Tests for ATP Formation. The tests were done in 1.8-ml microcentrifuge tubes, kept in ice water until ready for incubation. All reagents were prepared in SPK. To each tube were added 0.1 ml of one or two reagents, as indicated, or SPK in their place, and 0.2 ml of cell preparation, to a final volume of 0.4 ml. When incubated, the specimens were placed in a water bath for 1 hr at 34°C and oscillated at moderate speed. The reaction was stopped by placing the tubes in ice water, adding 40 μl of 70% (wt/vol) perchloric acid and, after rapid mixing, adding 65 μl of 7.5 M KOH containing 0.05 M EDTA, a volume previously determined to neutralize the perchloric acid in the buffer used. After vigorous mixing followed by a 5-min wait for the precipitate

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: DNP, 2,4-dinitrophenol.
†To whom reprint requests should be addressed.

to form, the tubes were centrifuged at top speed in a Microfuge (Beckman, no. 12) for 5 min. The supernatant fluids were stored at −20°C until assayed for ATP.

ATP Assays. The buffer for ATP assays was 0.1 M glycylglycine, pH 7.8, containing 9 mM $MgCl_2$ and 5 mM KCl. Since the SPK buffer, which is most favorable for the metabolism of the ehrlichiae, interfered with the ATP measurements, the specimens were diluted 1:11 in the glycylglycine buffer. The reaction mixture was prepared in a 6 × 50 mm borosilicate glass microtube by the addition of 10 μl of specimen, then 100 μl of buffer, and finally 25 μl of the luciferin/luciferase mixture (Sigma FLE-50). The luciferin/luciferase mixture was prepared the day before by rehydrating the lyophilized stock at 4°C with 5 ml of sterile distilled water. Immediately after the addition of the enzyme, the contents were mixed on a Vortex mixer and the tube was placed in an ATP photometer chamber. The emitted light was read 7 sec after the enzyme addition. The ATP photometer was model Chem Glow-2 (SLM Instrument). Each specimen was assayed in triplicate

Each experiment was analyzed on the basis of a standard curve of at least four different concentrations of ATP diluted in SPK and treated in the same manner as the test specimens. This was done not only to determine the sensitivity of the test with a given enzyme preparation but also to correct for the effects of dilution and of the different reagents on the emitted light. The ATP values of the specimens were calculated from a regression line derived from the ATP standards and expressed as either nmol per mg of protein or pmol per tube.

Other Procedures. CO_2 production from glutamine or other substrates by the ehrlichial and rickettsial preparation was determined by using radiolabeled substrates, as described previously (7). The protein contents of the preparations were determined by the Bradford protein assay method (10) (Bio-Rad). Electron microscopy procedures were as described previously (7). Chemical reagents were obtained from Sigma, Calbiochem, New England Nuclear, and other commercial sources.

RESULTS

A significant increase in ATP was demonstrated with *E. sennetsu* and two strains of *E. risticii* when the cells were incubated for 1 hr at 34°C with glutamine. Since in a previous study (7) no significant difference was noted in the rate of glutamine metabolism among the three strains, in these experiments no effort was made to test them for ATP production in an entirely comparable manner. Instead, each experiment was designed to include a different set of controls and/or cells purified by a different procedure.

E. sennetsu. ATP amounts were measured (Table 1) in cells purified by Renografin gradient and cryopreserved. Glutamine was not included in the diluents used for purification or cryopreservation. Cells that were quickly diluted, as appropriate, with SPK without substrate and maintained at 0°C prior to testing contained barely measurable amounts of ATP. After incubation for 1 hr at 34°C without substrate, ATP was no longer detected. When the cells were incubated with 5 mM glutamine, a marked increase in ATP was obtained. The addition of 25 μM atractyloside, an inhibitor of adenine nucleotide translocase in mitochondria (11), but not in rickettsiae (12), in conjunction with glutamine, decreased the ATP to a small extent. The difference was not significant. The addition of 1 mM DNP, an inhibitor of oxidative phosphorylation in both mitochondria and bacteria, resulted in a somewhat greater decrease in ATP ($P < 0.05$).

The control in this experiment consisted of host cells, mechanically disrupted, from which the nuclei had been removed by low-speed centrifugation (210 × *g* for 10 min). The ATP levels were low in all cases and differences among groups were not significant.

***E. risticii*, Maryland Strain, and *R. typhi*.** The *E. risticii* cells were prepared by the same procedure as the *E. sennetsu* cells described above and the results in this experiment (Table 2) were comparable. The major differences were the more marked reductions by the two inhibitors of oxidative phosphorylation. Although DNP clearly was the more effective inhibitor, the action by atractyloside suggests that not all mitochondria were removed from the *Ehrlichia* preparation.

R. typhi had been grown in the yolk sac of chicken embryos and purified by the Renografin procedure. During purification and cryopreservation the cells were maintained in diluents containing glutamate, the substrate most actively metabolized by rickettsiae. Preparations of rickettsiae purified from yolk sac are generally freer from host components than are ehrlichiae grown in tissue culture. Since rickettsiae grow in the cytoplasm of their host cells and not in the phagosome (13), host membranes, invariably retained in purified preparations of actively metabolizing *Ehrlichia* (6, 7), are not seen in *R. typhi* preparations (13). Glutamate was removed from the rickettsial preparation by high-speed centrifugation, just before the experiment. As expected, the ATP levels in rickettsiae were considerably higher than those obtained with ehrlichiae. The addition of atractyloside resulted in a slight increase in the rickettsial ATP. The decrease of the ATP induced by DNP was quite limited. This is not surprising, since in a separate experiment (not shown), DNP proved to be an ineffective inhibitor of rickettsial glutamine metabolism, as measured by CO_2 production.

***E. risticii*, Illinois Strain.** The cells used in this experiment were first subjected to thorough digestion with trypsin and DNase and separated from host constituents by Percoll gradient centrifugation, which in some cases proved to be a more effective method of purification (unpublished data). Furthermore, the cells were maintained in diluents containing 1 mM glutamine throughout the purification procedure and cryopreservation. Glutamine was removed from the preparations just before the experiment. Three fractions were obtained from the Percoll gradient. Fraction 1 (density = 1.048–1.080 g/ml), obtained from the bottom of the gradient,

Table 1. ATP production by *E. sennetsu* and denucleated uninfected $P388D_1$ host cells

Addition*	Incubation†	ATP, nmol/mg protein‡	
		Ehrlichia	$P388D_1$
None	−	0.25 ± 0.06	0.25 ± 0.03
None	+	Not detected	0.16 ± 0.13
Glutamine	+	2.42 ± 0.65	0.17 ± 0.08
+ Atractyloside	+	1.99 ± 0.10	0.17 ± 0.02
+ DNP	+	1.25 ± 0.02	0.19 ± 0.03

*Glutamine, 5 mM; atractyloside, 0.025 mM; 2,4-dinitrophenol (DNP), 1 mM.
†For 1 hr at 34°C. Specimens that were not so incubated were maintained for approximately 1 hr at 0°C.
‡Protein content: *Ehrlichia*, 0.155 mg per tube; $P388D_1$, 0.34 mg per tube. Mean ± SD of triplicate samples, each tested in triplicate.

Table 2. ATP production by *E. risticii* (Maryland strain) and *R. typhi*

Addition*	Incubation*	ATP, nmol/mg protein*†	
		Ehrlichia	*Rickettsia*
None	−	0.20 ± 0.02	4.26 ± 0.16
None	+	0.10 ± 0.01	1.04 ± 0.05
Glutamine	+	2.45 ± 0.16	11.68 ± 2.44
+ Atractyloside	+	1.00 ± 0.16	13.06 ± 1.11
+ DNP	+	0.46 ± 0.07	9.47 ± 1.40

*See footnotes of Table 1.
†Protein content per tube in both cases: 0.22 mg.

consisted of cells with greatest electron density and smallest contamination with host constituents (Fig. 1*A*), although some host membranes were retained (Fig. 1*B*). These cells also had the highest rate of glutamine metabolism (Table 3). Fraction 2 (1.044–1.048 g/ml), obtained from the intermediate section of the gradient, contained some *Ehrlichia* particles that were less electron dense and numerous empty vesicles and other structures, presumably of host origin (Fig. 1*C*). Glutamine specific activity was considerably lower. Fraction 3 (1.020–1.044 g/ml) is the top section of the gradient, which is generally discarded. Although some *Ehrlichia* particles are undoubtedly trapped, most of the other structures appear to be of host origin (Fig. 1*D*). Glutamine specific activity was quite low.

The ATP determinations of the three fractions (Table 3) reveal some similarities and some differences from those obtained in the previous two experiments (Tables 1 and 2). The amount of ATP in fraction 1 prior to incubation was considerably higher than the amounts obtained previously. There is a remarkable drop to a nonmeasurable level upon incubation without substrate. Upon incubation with glutamine there was an increase over the initial amount comparable to that obtained in the other experiments. Atractyloside had no apparent effect, while DNP was clearly inhibitory.

Table 3. ATP production by Percoll fractions separating *E. risticii* (Illinois strain) from host constituents

Addition*	Incubation*	ATP, nmol/mg protein		
		Fraction 1†	Fraction 2†	Fraction 3†
None	–	1.57 ± 0.22	0.37 ± 0.07	0.20
None	+	Not detected	0.02	0.20
Glutamine	+	3.73 ± 0.11	0.76 ± 0.15	0.27
+ Atractyloside	+	3.74 ± 0.25	0.77 ± 0.02	0.24
+ DNP	+	1.62 ± 0.08	0.37 ± 0.05	0.20

*See footnotes of Table 1.

†Fractions 1, 2, and 3 corresponded, respectively, to the bottom, intermediate, and top fractions of the Percoll gradient described in the text. Their appearance in the electron microscope is illustrated in Fig. 1. Protein contents were 0.11, 0.11, and 0.22 mg per tube, respectively. Glutamine metabolism (determined in a separate experiment), expressed as μmol of CO_2 per mg of protein produced from 5 mM glutamine, were 0.41, 0.12, and 0.02 per hr.

The results obtained with fraction 2 were qualitatively similar to those obtained with fraction 1, but much lower. They are compatible with the view that fraction 2 contained, proportionately, a much smaller number of *Ehrlichia* cells or cells of

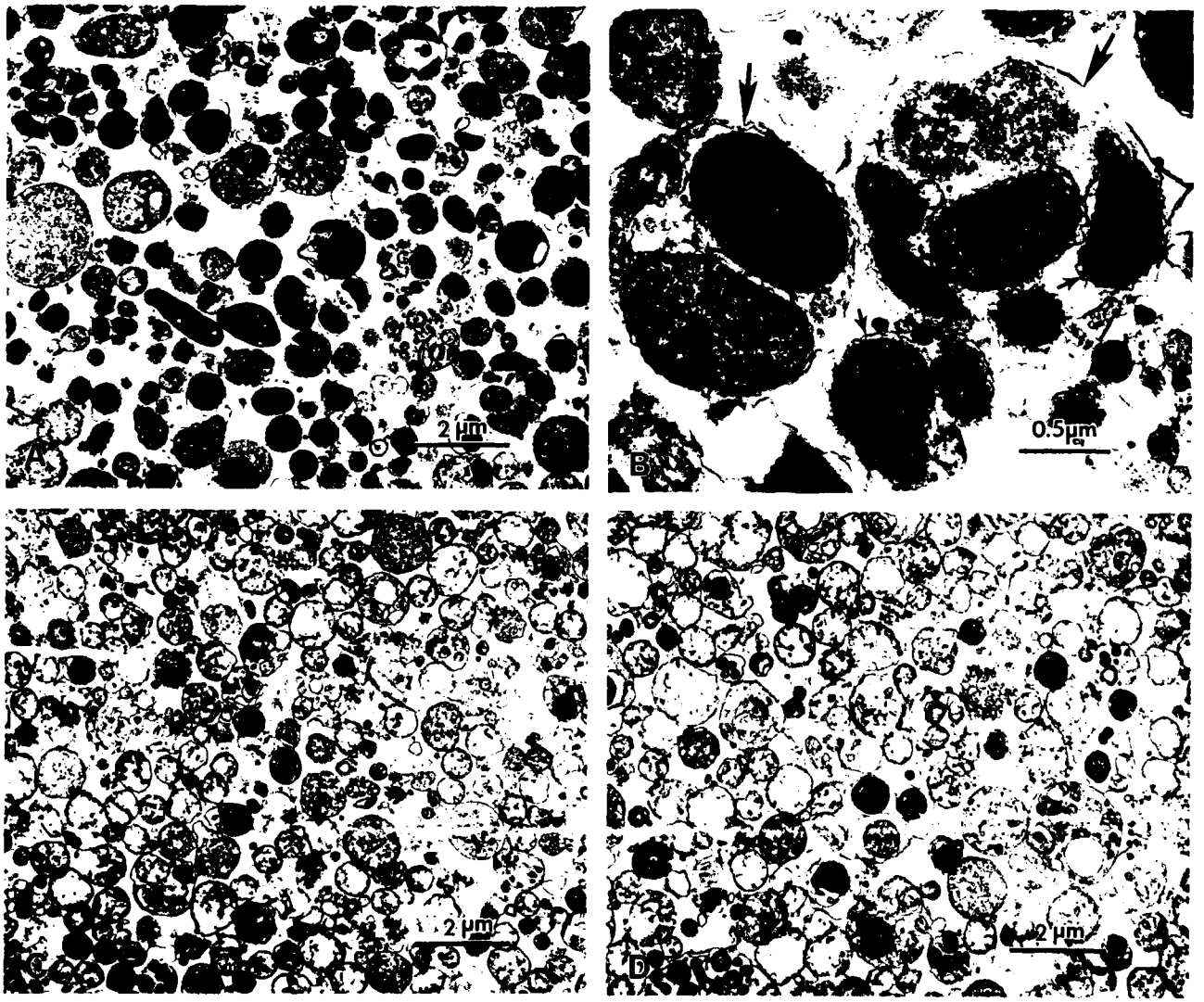

FIG. 1. Transmission electron micrographs of fractions 1, 2, and 3 of Percoll-purified preparation of *E. risticii* (Illinois strain) described in the text, and tested as shown in Table 3. (*A*) Fraction 1. (× 6800.) (*B*) Fraction 1, field adjacent to the one shown in *A*. Large and small arrows point, respectively, to what appear to be host and bacterial membranes. (×27,000.) (*C* and *D*) Fractions 2 and 3, respectively. (×6800.)

Table 4. ATP recovery after addition to preparations of uninfected denucleated P388D$_1$ cells, to purified *R. typhi*, and to Percoll fractions separating *E. risticii* (Illinois strain) from host constituents*

ATP added, pmol per tube	Incubation	ATP recovered, pmol per tube				
		P388D$_1$ (Table 1)	*R. typhi* (Table 2)	*E. risticii* fractions (Table 3)		
				1	2	3
0	−	84 ± 11	929 ± 35	177 ± 14	41 ± 8	44 ± 3
	+	52 ± 42	226 ± 11	0	2	43 ± 15
400	−	82 ± 12	1187 ± 89	445 ± 25	86 ± 0	51 ± 5
	+	78 ± 35	280 ± 31	7 ± 7	16 ± 0	36 ± 7
1200	−	85 ± 3	Not done	1001 ± 110	278 ± 50	125 ± 26
	+	45 ± 20	Not done	24 ± 14	16 ± 4	43 ± 7

*See footnotes of Tables 1–3.

reduced metabolic activity. The results obtained with fraction 3 were virtually identical to those obtained with denucleated uninfected cells (Table 1).

ATP Catabolism. The results illustrated in Table 3 with fraction 1 suggest that ehrlichiae catabolize ATP, thereby decreasing the internal ATP concentrations when the cells are incubated without substrate. In the experiments described above, we also attempted to determine if ATP degradation could be shown when ATP itself was added to the cells. An unexpected difficulty encountered in such determinations was that, although ATP was stable in the diluent used, it rapidly disappeared when added to control preparations, even before incubation. Table 4 illustrates results obtained with several of the preparations used in the experiments depicted in Tables 1–3. The results, in contrast to those previously presented, are given as pmol of ATP per tube, rather than nmol per mg of protein, to better correlate the amounts of ATP added to those recovered. With uninfected denucleated P388D$_1$ cells, the amounts of ATP were uniformly moderately low, irrespective of amounts of ATP added (0, 400, or 1200 pmol per tube). These amounts were not substantially changed by incubation for 1 hr at 34°C. By contrast, when 400 pmol per tube was added to *R. typhi*, before incubation the amounts of ATP were higher than those in comparable tubes to which no ATP had been added. After incubation the amounts of ATP in the two groups were about the same. Somewhat similar results were obtained with fraction 1 of *E. risticii*. The additions of 400 or 1200 pmol per tube were reflected in higher ATP levels before incubation. Surprisingly, the ATP levels after incubation were lower than those obtained with the P388D$_1$. With fraction 3 the results were similar to those obtained with P388D$_1$. The results with fraction 2 appeared to be intermediate between those with fractions 1 and 3, although more closely resembling those of fraction 3. With the other two *Ehrlichia* preparations (Tables 1 and 2), possibly because of less complete separation from host cell debris, the results were intermediate between those for fractions 1 and 2 (not shown).

It is obvious that the kinetics of ATP destruction by eukaryotic debris (P388D$_1$ and fraction 3) and those by ehrlichial or rickettsial preparations are quite different. Whether the destruction of ATP by ehrlichiae was due to an ATPase or some other enzyme, such as a phosphatase, kinase, or pyrophosphatase, has not yet been investigated. The results are consistent with the expectation that ehrlichiae can catabolize extracellular ATP, although a role by host enzymes in these reactions cannot be excluded. It remains to be shown whether ATP is also transported intact by *Ehrlichia*, as found for typhus rickettsiae (12).

DISCUSSION

Our results clearly demonstrate that two monocytic *Ehrlichia*, *E. sennetsu* and *E. risticii*, are capable of limited ATP synthesis *in vitro* when incubated with glutamine. It is tempting, therefore, to compare this and other biological properties of ehrlichiae with those of other obligate intracellular bacteria. Our understanding of the biological properties of ehrlichiae, however, has not kept pace with our expanding knowledge of ehrlichiae as agents of human and animal diseases (1, 7). For example, casual examination of electron micrographs of ehrlichiae (Fig. 1 *A* and *B*; refs. 4, 5, 7, 14, and 15) would tend to link them to chlamydiae (16, 17). Ehrlichiae, as well as chlamydiae, multiply in the phagosomes of host cells and during growth are surrounded by a host cell membrane. There is good evidence that chlamydiae have surface components that inhibit fusion of the phagosome with lysosomes (16). Such a mechanism may exist in ehrlichiae, but it has not yet been demonstrated. The only biochemical evidence supporting the intraphagosomal location of ehrlichiae is the pH optimum for metabolic activity, which is moderately alkaline (7). In addition, electron micrographs of ehrlichiae clearly display forms varying in size and electron density that suggest the existence of a developmental cycle. In chlamydiae a cycle was clearly demonstrated with differentiation into elementary and reticulate bodies. The elementary bodies are infectious, inhibit phagosome–lysosome fusion, and are toxic for mice and macrophages. The reticulate bodies have none of these properties (16) but, in contrast to elementary bodies, are capable of metabolic activity and multiplication (17). There is no indication that such a clear differentiation exists in ehrlichiae, but the cycle of development of ehrlichiae has not been subjected to critical scrutiny. Another characteristic of chlamydiae, which is quite uncommon among bacteria (18), is the lack of a peptidoglycan layer (17). Although ehrlichiae may have low amounts of peptidoglycan and lipopolysaccharide, as is the case in *Rickettsia tsutsugamushi* (19), the outer membranes of ehrlichiae have not been studied in sufficient detail to permit such a comparison.

The main difference between ehrlichiae and rickettsiae is the location of their intracellular growth, since rickettsiae multiply in the cytoplasm of their host cells without being surrounded by a host membrane (13). This location is attributed to the stimulation of phospholipase A activity, which permits the rickettsia to escape from the phagosome as it induces its own phagocytosis (20). Biochemically, ehrlichiae are otherwise more closely akin to rickettsiae than to chlamydiae. Although experiments identical to those reported here, to our knowledge, have not been performed with chlamydiae, there is good evidence that they are totally dependent on exogenously supplied ATP (17, 21). Ehrlichiae resemble rickettsiae in the type of substrate they utilize (7) and ATP formation *in vitro*.

In conclusion, the similarity between the monocytic ehrlichiae and the rickettsiae in metabolic activity contrasts with the difference in the nature of their intracellular parasitism. The examination of the 16S rRNA sequences of *Rickettsia*

prowazekii and *E. risticii* showed that *E. risticii* is specifically related to the rickettsia in the α subdivision of the purple bacteria (reviewed in ref. 22). Chlamydiae, on the other hand, are not closely related to any of the other eubacteria and are only distantly related to the planctomyces and related species (23). These results tend to indicate that the difference in intracellular location between the ehrlichiae and rickettsiae is not of paramount phylogenetic importance. Other factors, such as ecology in mammalian and, presumably, arthropod hosts (1), might be more significant.

Note Added in Proof. The inhibition of phagosome–lysosome fusion by ehrlichiae has recently been demonstrated (24).

We are greatly indebted to Nancy A. Robinson and Judith G. Pace of the Pathophysiology Division of the U.S. Army Medical Research Institute for Infectious Diseases for their instruction in the use of the equipment for ATP assay and to Robert Williams of the Naval Medical Research Institute for technical assistance in the electron microscopy preparations. This work was supported by the Naval Medical Research and Development Command, Department of the Navy, Research Project 3M161102.BS10.AK.111, and the U.S. Army Medical Research Institute for Infectious Diseases.

1. Ristic, M. (1986) in *Microbiology-1986*, ed. Leive, L. (Am. Soc. Microbiol., Washington, DC), pp. 182–187.
2. Misao, T. & Kobayashi, Y. (1955) *Kyushu J. Med. Sci.* **6**, 145–162.
3. Rapmund, G. (1984) *J. Infect. Dis.* **149**, 330–338.
4. Holland, C. J., Ristic, M., Cole, A. I., Johnson, P., Baker, G. & Goetz, T. (1985) *Science* **227**, 522–524.
5. Holland, C. J., Weiss, E., Burgdorfer, W., Cole, A. I. & Kakoma, I. (1985) *Int. J. Syst. Bacteriol.* **35**, 524–526.
6. Weiss, E., Dasch, G. A. & Kang, Y. (1985) in *Rickettsiae and Rickettsial Diseases*, ed. Kazar, J. (Slovak Academy of Science, Bratislava, Czechoslovakia), pp. 38–45.
7. Weiss, E., Dasch, G. A., Kang, Y. & Westfall, H. N. (1988) *J. Bacteriol.* **170**, 5012–5017.
8. Weiss, E. (1973) *Bacteriol. Rev.* **37**, 259–283.
9. Williams, J. C. & Weiss, E. (1978) *J. Bacteriol.* **134**, 884–892.
10. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 238–254.
11. Bruni, A., Contessa, A. R. & Luciani, S. (1962) *Biochim. Biophys. Acta* **60**, 301–311.
12. Winkler, H. H. (1976) *J. Biol. Chem.* **251**, 389–396.
13. Weiss, E. (1982) *Annu. Rev. Microbiol.* **36**, 345–370.
14. Ristic, M. & Huxsoll, D. L. (1984) in *Bergey's Manual of Systematic Bacteriology*, eds. Krieg, N. R. & Holt, J. G. (Williams & Wilkins, Baltimore), pp. 704–709.
15. Rikihisa, Y. (1986) in *Microbiology-1986*, ed. Leive, L. (Am. Soc. Microbiol., Washington, DC), pp. 200–202.
16. Moulder, J. W. (1985) *Microbiol. Rev.* **49**, 298–337.
17. Moulder, J. W. (1984) *ASM News* **50**, 353–362.
18. Woese, C. R. (1987) *Microbiol. Rev.* **51**, 221–271.
19. Amano, K., Tamura, A., Ohashi, N., Urakami, H., Kaya, S. & Fukushi, K. (1987) *Infect. Immun.* **55**, 2290–2292.
20. Winkler, H. H. (1986) *Ann. Microbiol. (Paris)* **137A**, 333–336.
21. Weiss, E. & Wilson, N. N. (1969) *J. Bacteriol.* **97**, 719–724.
22. Weisburg, W. G. (1989) in *Intracellular Parasitism*, ed. Moulder, J. W. (CRC, Boca Raton, FL), in press.
23. Weisburg, W. G., Hatch, T. P. & Woese, C. R. (1986) *J. Bacteriol.* **167**, 570–574.
24. Wells, M. Y. & Rikihisa, Y. (1988) *Infect. Immun.* **56**, 3209–3215.