INCORPORATION OF ACETATE-1-C^{14} INTO LIPID BY TYPHUS RICKETTSIAE[1]

MARIANNA R. BOVARNICK

Radioisotope and Laboratory Services, Veterans Administration Hospital, Brooklyn, and the Department of Medicine, State University of New York Downstate Medical Center, Brooklyn, New York

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Although it has been known for some time that rickettsiae have an independent, if limited, oxidative metabolism (Bovarnick and Snyder, 1949), evidence of any synthetic activity on the part of these organisms has until recently been lacking. It was noted (Bovarnick, 1956) that they were capable through oxidative phosphorylation of forming ATP,[2] a compound that appears to be a prerequisite for all known biological syntheses. In spite of this ability it has not yet been possible to detect directly the synthesis of measurable amounts of any substance by viable rickettsiae. However, in the past year Myers, Paretsky, and Downs (1959) have found that sonic extracts of *Coxiella burnetii* can form serine from glycine and formaldehyde, and Bovarnick Schneider, and Walter (1959) and Fujita, Kohno, and Shishido (1959) have found that viable preparations of *Rickettsia prowazekii* and *Rickettsia mooseri* can incorporate trace quantities of isotopically labeled amino acids. Some of the conditions necessary for this last process indicate that the incorporation may be due to a small amount of protein synthesis. Recently we have also observed incorporation of acetate into lipid by typhus rickettsiae and, despite difficulties in determining the optimal conditions for incorporation, the data suggest that rickettsiae can carry out a third type of synthetic reaction.

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[2] The following abbreviations are used: GSH, reduced glutathione; DPN and DPNH, the oxidized and reduced forms of diphosphopyridine nucleotide; TPN and TPNH, the oxidized and reduced forms of triphosphopyridine nucleotide; CoA, coenzyme A; ADP and ATP, the di- and triphosphates of adenosine.

EXPERIMENTAL METHODS

The E strain of typhus rickettsiae, grown in the yolk sacs of embryonated eggs, was purified as described elsewhere (Bovarnick, 1956). The incorporation of acetate-C^{14} by these purified rickettsiae was measured as previously described for determination of the uptake of methionine-S^{35} (Bovarnick et al., 1959). The composition of the basal medium used in these experiments, a modification of the one used in the study of amino acid incorporation and probably much more complicated than necessary, is given in table 1. Because of the relatively low uptakes observed with acetate, it was necessary to increase the concentration of rickettsiae during incubation to 0.3 mg protein per ml, and to increase the size of the samples taken for assay to 4 ml. With samples of this size it was not necessary to add carrier rickettsiae before isolation of the labeled rickettsiae for analysis. Uptakes are given as counts:min:mg rickettsial protein and are of the same order of magnitude as the actual counts measured. An uptake of 100 counts/min represents incorporation of 0.11 mμmole acetate.

For preparation of glycine-C^{14} labeled rickettsiae, the organisms were incubated at a concentration of 30 μg rickettsial protein per ml for 24 hr at 30 C in the medium previously described (Bovarnick and Schneider, 1960*a*), except that glycine-2-C^{14}, with a specific activity of 5 mc/mmole, obtained from Nuclear-Chicago Corporation, was used in place of the glycine-1-C^{14} used earlier.

RESULTS AND DISCUSSION

The amount of acetate incorporated after 5 or 24 hr incubation at 30 C was very small, at least an order of magnitude lower than the amount of amino acid incorporated by these organisms under similar conditions. In the two

TABLE 1
Composition of medium

Constituent	Final Concn	Constituent	Final Concn
Amino Acids	μM	*Salts*	mM
DL-Alanine	95	KCl	105
L-Arginine	48	K_2HPO_4	3.9
L-Asparagine	48	KH_2PO_4	1.9
L-Cysteine	19	$MgCl_2$	0.86
Glycine	48	$MnCl_2$	0.046
L-Histidine	19	$CaCl_2$	0.012
L-Proline	48	$FeCl_3$	0.008
L-Hydroxyproline	48	*Cofactors*	
L-Serine	48	DPN	0.38
L-Threonine	71	DPNH	0.19
L-Tryptophan	7.1	TPN	0.03
L-Tyrosine	48	TPNH	0.06
L-Valine	71	Coenzyme A	0.035
L-Leucine	71	Cocarboxylase	0.055
L-Isoleucine	95	GSH	1.0
L-Lysine	71	ATP	1.0
L-Methionine	24	Guanosine triphosphate	0.083
L-Phenylalanine	38	Uridine triphosphate	0.084
L-Aspartic acid	48	Cytidine triphosphate	0.085
L-Glutamine	5000	Glycerophosphate	0.29
Vitamins		Phosphocholine	0.14
Choline chloride	4.6	Phosphoethanolamine	0.11
Folic acid	0.036	Phosphoserine	0.11
Hemin chloride	0.10		
Inositol	3.2	Na-acetate-1-C^{14}	0.008
Biotin	0.32		
p-Aminobenzoic acid	1.0	pH 7.0–7.2	
p-Hydroxybenzoic acid	1.3		
Vitamin B_{12}	0.032		
Leucovorin	0.036		
DL-α-Lipoic acid	2.0		
	mg/ml		
Yolk sac protein	0.29		

The medium was sterilized as described by Bovarnick et al. (1959) and the soluble yolk sac fraction was prepared as described by Bovarnick and Schneider (1960*a*).

The acetate-1-C^{14} was obtained from Nuclear-Chicago Corporation with a stated specific activity of 5 mc/mmol. At the concentration used in these experiments the measured activity was 7,200 counts:min:ml.

experiments given in table 2 the maximal specific activities attained corresponded to incorporation of 2.4 and 14 μg acetic acid per gram of rickettsial protein, respectively. However, even though low, the observed uptake appeared to be significant, since uptake by heated rickettsiae or by particles from normal yolk sacs was lower by one or two orders of magnitude and probably insignificant.

In an effort to determine the nature of the substances labeled after incorporation of acetate-C^{14}, the labeled rickettsiae were fractionated and the distribution of activity between the different fractions was compared with that found after incorporation of glycine-C^{14} (table 3). It is quite apparent that after incubation of rickettsiae with acetate-C^{14} the incorporated radioactivity is found almost completely in the lipid, whereas after incubation with glycine-C^{14} it appears chiefly in the protein. It should be mentioned that the reason for the similarity in the initial specific activities of the acetate and glycine labeled rickettsiae is that carrier rickettsiae were added immediately after incubation to those that had incorporated glycine, the ratio of carrier to labeled rickettsiae being about 20:1, but none was added to those that had incorporated acetate. The relatively low recovery of counts in the case of the glycine-C^{14} labeled rickettsiae is probably due to the frequent poor separation of the small precipitates on centrifugation. It was generally necessary to filter all supernatants to obtain completely clear solutions, and recovery of the small amounts of insoluble material, probably largely protein, that was lost on the filters was not attempted.

Although qualitatively the rickettsiae have always been found capable of incorporating labeled acetate, quantitatively the amount of incorporation in different experiments has varied widely. Also the response of the uptake to certain alterations in the conditions of incubation has been variable, partly because the effect of some single constituents of the medium appears to depend upon the presence or absence of others. Since the factors determining this variability have not yet been worked out, detailed presentation of the results does not seem justified, but certain generalizations may be made. Glutamine or glutamate and GSH appear consistently to

TABLE 2

Incorporation of acetate-1-C^{14} by typhus rickettsiae

Expt No.	Time of Incubation	Incorporation of Acetate-C^{14} by		
		Rickettsiae	Heated rickettsiae*	Normal yolk† sac particles
	hr	*counts/min*		
1	5	36	1	2
2	5	123	3	3
	24	215		5

The figures for counts per minute represent counts incorporated per mg rickettsial protein or yolk protein.

* These rickettsiae were heated for 15 min at 56 C before addition to the medium for incubation.

† Particles from normal yolk sacs, which had been harvested from unifected eggs of the same age as the infected eggs, were prepared by the same procedure as used for preparation of the rickettsiae, except that the last cycle of alternate high and low speed centrifugation was omitted to avoid loss of almost all normal yolk sac material. The normal yolk sac particles were suspended in a volume such that their protein content would be approximately the same as that of the rickettsial preparations. The volume was ⅓ to ¼ that used for rickettsiae from an equivalent quantity of starting yolk sac homogenate.

TABLE 3

Distribution of radioactivity in rickettsiae labeled with acetate-1-C^{14} or with glycine-C^{14}

Fraction	Radioactivity of Rickettsiae Labeled with			
	Glycine-C^{14}		Acetate-C^{14}	
	total counts/min	*counts: min:mg protein*	*total counts/min*	*counts: min:mg protein*
Initial	1,085	199	1,320	94
Cold perchloric acid soluble	21		33	
Lipid	6		1,110	
Lipid residue	40	226	22	15
Hot perchloric acid soluble	118		70	
Protein	718	197	55	6

The trichloroacetic acid insoluble fraction of rickettsiae that had been incubated for 24 hr in a medium containing the labeled compound was first isolated as usual, i.e., after heating for 15 min at 56 C and addition of carrier glycine or acetate, the mixtures were centrifuged and the precipitates were washed twice with 5 per cent trichloroacetic acid, once with water, then dissolved in 0.003 M KOH. This material represents the initial preparations. A sample of this was removed for counting and protein determination (Lowry et al., 1951) and the remainder was precipitated by addition of perchloric acid to a final concentration of 2.5 per cent. The precipitate was extracted once with 3:1 ethanol-ether at 56 C for 20 min, once with 1:1 ethanol-ether, and once with ether. The combined alcohol ether extracts were dried, extracted with chloroform, the extract filtered, dried, and counted. The chloroform extract is designated the lipid fraction. The alcohol ether soluble, chloroform insoluble material was dissolved in 0.003 M KOH before drying for counting and is designated the lipid residue. It contained a small amount of protein, possibly from lipoproteins. The alcohol ether insoluble material was extracted twice for 20 min at 70 C with 5 per cent perchloric acid to remove nucleic acids, then washed once with water, and finally resuspended in 0.003 M KOH. This is designated the protein fraction. The hot and cold perchloric acid extracts were separately filtered, neutralized with KOH, chilled, centrifuged to remove insoluble $KClO_4$, and concentrated to a small volume.

be needed for good uptake and the reduced pyridine nucleotides usually bring about a 20 to 50 per cent increase in uptake. Many of the other constituents of the medium, i.e., the amino acids other than glutamine, the vitamins, and probably phosphocholine, phosphoethanolamine, phosphoserine, and phosphoglycerate, as well as bicarbonate, which is frequently of importance in lipid synthesis in other systems, appear to be without effect. The response of acetate incorporation to added purine and pyrimidine nucleotides has been particularly variable. The mononucleotides have been consistently without effect, but ATP and the other nucleoside triphosphates have shown effects varying from a 2-fold increase in the amount of acetate incorporated to an 80 per cent decrease. There is some indication that the effect of ATP (and ADP) varies with the concentration of rickettsiae during incubation, also with the concentration of the other nucleoside triphosphates, and that it can be altered by suitable pretreatment of the rickettsiae. The effect of the other nucleoside triphosphates in turn appears to depend at least in part on the concentration of ATP. It is of interest in this connection that one other reaction brought about by typhus rickettsiae, the lysis of sheep erythrocytes, also shows great varration in

response to added ATP. The factors bringing about the variations in the case of the hemolytic reaction can be controlled and have been described elsewhere (Bovarnick and Schneider, 1960*b*).

It is probably not surprising that the uptake of acetate-C^{14} by typhus rickettsiae is small, for glutamate, which they rapidly form from the glutamine present in the medium (F. E. Hahn, *unpublished observation*), is oxidized by these organisms via the tricarboxylic acid cycle (Wisseman et al., 1952; Bovarnick and Miller, 1950; Price, 1953; Paretsky et al., 1958). Therefore acetyl-CoA, which in all known systems is a more immediate lipid precursor than acetate itself, is presumably continuously being formed from the unlabeled glutamine as well as from the labeled acetate. It had at first been hoped that this difficulty could be avoided by omitting glutamine and providing ATP and the reduced pyridine nucleotides as substitutes. While a very small uptake was observed under such conditions, much better uptake was obtained in the presence of glutamine, despite the probable dilution of isotopic acetate thereby introduced, probably because glutamate is the best source of energy for whole rickettsiae (Bovarnick and Allen, 1957). The extent of the dilution introduced by glutamate oxidation cannot be evaluated, since it is not known whether acetyl-CoA formed during this reaction and that formed from externally added acetate are equivalent as lipid precursors, or whether one or the other is used preferentially. The rate of oxidation of glutamate, about 25 μl O_2:hr:mg rickettsial protein at 30 C (Bovarnick and Schneider, 1960*b*) would, if the oxidation were complete, be equivalent to the oxidation of 0.075 μmoles glutamate: hr:ml at the concentration of rickettsiae used in these experiments. Since the oxidation is not usually quite complete (Bovarnick and Miller, 1950), the potential rate of acetyl-CoA formation would be lower than this, but still probably sufficient to produce fairly extensive dilution of the acetyl-CoA formed from the added acetate (0.008 μmoles/ml) during the 5- to 24-hr incubation periods, if there were complete equilibration of acetyl-CoA formed from the two sources. However equilibration cannot be complete, since decreasing the concentration of rickettsiae, and hence the rate of oxidation of glutamate, has little effect on the amount of labeled acetate incorporated per mg rickettsial protein. The extent of the dilution must therefore depend on the relative availability and rates of formation of acetyl-CoA from glutamate and from external acetate. Variation in the amount of added labeled acetate incorporated may be produced by any factors that affect the relative utilization of the two sources of acetyl-CoA, as well as by variation in the amount of lipid formed. Because of these complications this system may be inherently a poor one for reliable estimation of the amount of lipid formed from the amount of incorporation and further attempts to improve the quantitative reproducibility of acetate incorporation into lipid by whole viable rickettsiae may not be warranted. The data do nonetheless seem worth recording in that they indicate in a qualitative fashion that rickettsiae are capable of lipid synthesis and that the very simple substrate acetate is one possible precursor for this synthesis.

SUMMARY

Typhus rickettsiae, incubated in a medium similar to that used for demonstration of amino acid incorporation, are capable of incorporating acetate-C^{14}. The amount of acetate incorporated is small but is in marked contrast to the practically negligible uptake of acetate by heated rickettsiae or by particles from normal yolk sac. The incorporated acetate-C^{14} is found exclusively in the lipid fraction of the cells, in contrast to incorporated glycine-C^{14}, which is found almost entirely in the protein fraction.

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