HOST-PARASITE METABOLIC INTERRELATIONSHIPS: THE METABOLISM OF ACETATE-U-C^{14} AND RIBOSE-1-C^{14} IN CHICK EMBRYOS INFECTED WITH RICKETTSIAE TYPHI

by

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A Thesis Submitted to the Faculty of the

DEPARTMENT OF MICROBIOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

MASTER OF SCIENCE

In the Graduate College

THE UNIVERSITY OF ARIZONA

1965

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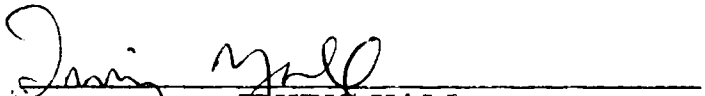

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Date

ACKNOWLEDGMENTS

The author wishes to express his thanks to Dr. Irving Yall and Dr. Kenneth Wertman for their assistance and encouragement during this study.

The deepest appreciation is expressed to my parents, Mr. and Mrs. C. de Wolfe, without whose assistance, understanding and love this work would have been impossible.

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ABSTRACT

In this study acetate-U-C^{14} and ribose-1-C^{14} were employed to determine the effects of infection with Rickettsiae typhi upon the metabolism of the embryonated chicken egg.

Respired carbon dioxide was assayed for radioactivity content and egg and rickettsial fractions were extracted and treated in such a manner as to isolate and identify radioactive compounds in these fractions.

In the acetate study it was found that infection with R. typhi apparently forces the egg to utilize this substrate for lipid synthesis which is recycled through the tricarboxylic acid cycle along with acetate metabolized directly by this means.

In the studies employing ribose as a substrate it was found that the rickettsiae did not incorporate this compound into nucleic acids. It has been hypothesized that the chicken embryos utilize the pentose pathway in the metabolism of this carbohydrate.

INTRODUCTION

The study of the metabolism of the rickettsiae has been a long, slow process. Contributions made since World War I have made possible intensive studies into the mechanisms and relationships of host-parasite metabolism.

The importance of a few significant discoveries cannot be underestimated. They include the demonstration by Cox (1938, 1941) that the yolk sac of the intact, developing chick embryo can be successfully employed for the propagation of rickettsiae. The relatively recent introduction of cellulose anion exchange columns by Hoyer, et al. (1958) and the cation exchange resin by Hara et al. (1958) for the recovery of highly purified rickettsiae has made the extraction of the cellular components of these organisms possible.

Another valuable contribution by Bovarnick, Miller and Snyder (1950) was the introduction of sucrose-potassium-glutamate broth for the satisfactory storage of rickettsiae.

In vitro metabolic studies have been carried out on a wide scale since the early 1950's, and many rickettsial metabolites and enzyme systems have been identified.

Bovarnick and Snyder (1949) demonstrated oxygen up-take by suspensions of living epidemic and murine typhus rickettsiae in the

presence of glutamate, laying the ground work for subsequent studies of metabolic activities of rickettsiae. Bovarnick et al. (1950) showed the presence of rickettsial glutamic transaminase, and on the basis of these studies, they suggested that rickettsial oxidation of glutamate proceeded through the citric acid cycle.

Wisseman, et al. (1952) demonstrated that glutamic oxidation was affected by enzyme inhibitors but not by rickettsial inhibitors. Wisseman et al., (1952) hypothesized an initial oxidative deamination to form alpha-keto glutamic acid which would then proceed through all of the steps of the conventional Krebs cycle.

Bovarnick and Allen (1953), Gilford and Price (1955) and Rosenberg and Bovarnick (1954) demonstrated that diphosphopyridine nucleotide (NAD) and coenzyme A (CoA) were essential to the metabolic integrity of rickettsiae.

Bovarnick (1956) demonstrated that typhus rickettsiae utilize phosphate to form adenosine triphosphate from adenosine diphosphate during the oxidation of glutamate, and that the process is enhanced by the addition of the co-factors, NAD and CoA. Bovarnick and Allen (1957), demonstrated the progressive loss of adenosine triphosphate during starvation of rickettsiae.

Hopps, et al., (1956) demonstrated that R. rickettsia contained an intrinsic glutamic-aspartic transaminase, and Hayes, et al., (1957) demonstrated electron transport by a flavin-enzyme-iron-cytochrome

system in the same organism.

Paretsky et al., (1958) reported that R. burnetii possessed an ADP-dehydrogenase, and could synthesize citrate from acetate or acetyl phosphate in the presence of oxalacetate. Hahn, et al., (1959) demonstrated the ability of R. rickettsia to deaminate glutamine to glutamic acid and likewise, asparagine to aspartic acid.

It must be noted that these in vitro studies are not necessarily analogous to the reactions of rickettsiae in vivo, and until the work of Beakley (1962) and Swack (1965), the in vivo metabolism of these organisms had not been studied.

Beakley reported that infection of embryonated eggs with R. typhi brought forth an alteration of the metabolic pathways of the egg, causing a greater utilization of the hexose-monophosphate pathway. This was shown by the fact that most of the label from glucose-6-C^{14} introduced into the yolk sac of infected eggs was recovered in an RNA-like substance isolated from the rickettsia, and that there was a significant rise in the ratio of label recovered from glucose-1-C^{14} carbon dioxide over that of the glucose-6-C^{14}.

Swack demonstrated that infection with R. typhi causes the metabolism of embryonated chicken eggs to shift toward increased synthesis of nucleic acid. He proved this by demonstrating that the ribonucleic acid labeled with carbon-14 and phosphorus-32 extracted from infected tissue had a specific activity significantly higher than

uninfected controls. The same was reported for purine and pyrimidine bases resolved from the ribonucleic acid and deoxy-ribonucleic acid.

The purpose of this research project is to expand the knowledge of *in vivo* metabolic relationships between host (the egg) and parasite (*R. typhi*) utilizing the following procedures.

I. In the case of sodium acetate-U-C^{14} studies.

A. Determination of carbon dioxide release from infected and non-infected eggs in an attempt to correlate the over-all effect of infection on metabolism.

B. The distribution of the total radioactivity within the egg.

C. The labeling of rickettsiae and rickettsial fractions, including distribution within the various fractions.

II. In the case of ribose-1-C^{14} studies.

A. Determination of rickettsial uptake of the label and distribution of the label within the rickettsial fractions.

MATERIALS AND METHODS

The Host

Fertile hens' eggs from the University of Arizona white leghorn flocks were employed exclusively. Because of possible antirickettsial activity, only eggs from chickens fed antibiotic free feed were used. The eggs were collected and stored at 4 C for no more than three days before incubation. All eggs used in critical analytical procedures were weighed in an attempt to obtain a certain degree of uniformity.

Carbon dioxide collection experiments were carried out in a walk-in incubator held at 37 C. Experiments for purification, isolation and analysis of rickettsiae not including CO_2 data were carried out in a Humidaire egg incubator (Humidaire Incubator Co., 217 West Main Street, New Madison, Ohio) at 36 C and 70% relative humidity.

In the radioactivity experiments the egg inoculations were handled as follows: for the CO_2 collecting run, using sodium acetate-U-C^{14}, the eggs were allowed to incubate four days, they were then candled, and infertile eggs were discarded. At this time all fertile eggs were injected with the radioactive substrate (described elsewhere) on the fourth day. After six days of incubation, fertile eggs were inoculated as follows: One group was inoculated with 0.2 ml each of rickettsial suspension, a second with 0.2 ml each of sterile sucrose-

potassium glutamate (sucrose-P.G.) suspending medium (Bovarnick, Miller and Snyder, 1950), and a third group was only punctured with a sterile needle to compensate for traumatic shock of injection, this group was used as the uniform control. All inoculations were made through the air sac with sterile 21 gauge, one and one half inch needles, directly into the yolk of the egg.

For the experiment utilizing ribose-1-C^{14}, all inoculations were made on the sixth day of incubation utilizing only radioactive substrate and rickettsial suspension in the manner described above.

Before the isotope experiments were conducted, toxicity tests were performed with unlabeled sodium acetate and ribose to determine any possible toxic effects of the substrate, in both cases none were detected.

All infected eggs that died before the fourth day after infection were discarded, the eggs that survived until the sixth day after infection were treated as follows:

1) Sodium acetate-U-C^{14} experiments.

   a) Embryo - washed three times in sucrose- P.G., frozen, and stored at -20C.

   b) Yolk sac - washed three times in sucrose-P.G., and either frozen or subjected to a purification procedure described elsewhere.

c) Embryonic membrane - washed three times in sucrose-P. G., frozen, and stored at -20 C.

d) Chorioallantoic membrane - washed three times in sucrose-P. G. frozen, and stored at -20 C.

e) Yolk - consisting of all remaining egg material plus the washings of other fractions. This material was frozen, and stored at -20 C.

2) Ribose-1-C^{14} experiments.

a) The yolk sac was washed once in sucrose-P. G. and subjected to purification procedures (described elsewhere), all other egg fractions were discarded.

The Parasite

1) Preparation of the parasite.

A frozen suspension of Rickettsia typhi, Wilmington strain, was obtained from the stock culture collection of the Department of Microbiology, University of Arizona. This strain was originally obtained from The Department of Rickettsial Diseases, Walter Reed Army Institute of Research, Washington, D. C.

The rickettsial suspension was rapidly thawed, diluted to 20% with sucrose- P. G., and 0. 2 ml was injected into each of fifty eggs. The yolk sacs were harvested and the large pool of infected material was homogenized in a "dispersal" Waring blender to make a 20%

suspension of yolk sac in sucrose-P. G. The preparation was dispensed aseptically in 12 x 100 mm screw capped tubes and shell-frozen in -60 C. acetone and stored at -60 C. A tube of this suspension was rapidly thawed in warm water and diluted with sucrose-P. G. to a 10% suspension. Two-tenths ml of this preparation was used as the standard inoculum in all experiments.

2) Purification of the parasite.

A modification of the method of purification employed by Yamamoto et al., (1958) and Hara (1958) was utilized to separate the rickettsiae from the yolk sac.

a) Preparation of the ion exchange resin.

Four grams of the ion exchange resin, amberlite XE-64 (Rohm and Haas Co., Philadelphia 5, Pennsylvania) was mixed with 30 ml of 1 N NaOH, stirred thoroughly and centrifuged at 1,000 r.p.m. for 5 minutes. The sediment was mixed with 1.2% KCl, stirred, and centrifuged at 1,000 r.p.m. for 5 minutes. The sediment was again mixed with 1.2% KCl, stirred, and centrifuged for 30 minutes. The sediment was mixed with 10 volumes of sucrose-P. G. and stored at 4 C.

b) Purification procedure.

Infected yolk sacs were harvested, washed three times in sucrose-P. G. and blended with sucrose-P. G. for 60 seconds in 10 second increments in the Waring blender. This suspension was centrifuged at 11,000 r.p.m. for one hour. The sediment was mixed in

10 ml amberlite XE - 64 suspension, and centrifuged at 1,000 r.p.m. for 30 minutes. This step was repeated if the supernatant was cloudy. The supernatant was then centrifuged at 11,000 r.p.m. for 1 hour and the pellet was shell frozen and stored at -60 C.

3) Verification of purity and activity of rickettsial suspensions.

a) Two methods of verifying rickettsial purity were used and are as follows:

1) Guinea pig inoculations

Two male guinea pigs were inoculated with 0.2 ml each of the rickettsial suspension and fever charts prepared for each animal. The response was typical of that expected for murine typhus. The temperature reached a peak on the sixth to seventh day accompanied by extreme scrotal swelling. At this time the spleen and tunica vaginalis were removed aseptically, assayed for rickettsiae and stored at -60 C.

2) Machiavello's stain

All experiments utilizing rickettsiae were checked for the presence of the organism and absence of contaminants by utilizing the Machiavello stain (Grey, 1954). In all cases,

microscopic examination revealed large numbers of rickettsiae, and in the case of guinea pig tissues, Mooser bodies and intra-cytoplasmic rickettsiae were observed.

b) To check for activity of the rickettsial suspension and determine the most effective titer to utilize for egg infection, titrations (ID_{50}) were performed on six day old embryonated eggs utilizing the Reed and Muench (1938) method. It was found that yolk sac homogenate diluted to a 10-20% suspension with sucrose-P.G. was most effective in terms of infectivity and yield.

Radioactive Substrates

Substrates utilized in this study were sodium acetate labelled uniformly with carbon - 14 and D - ribose labelled with carbon - 14 at the number one position, both of which were obtained from Nuclear Chicago Corporation, Chicago 10, Illinois. Each substrate was dissolved in a minimum amount of distilled water, sterilized by filtration, aliquoted aseptically into sterile screw-capped tubes, and frozen until utilized.

The sodium acetate-U-C^{14} had a specific activity of 34 millicuries per millimole (manufacturer's data), and was shown to have the following radiochemical purity:

1) By dilution analysis as silver acetate ≦ 96%

2) By paper chromatography in

a) ethanol: ammonia:water ≦ 97%
b) n - butanol: ethanol: 3N ammonia ≦ 97%

The sodium acetate-U-C^{14} was supplied freeze-dried and sealed under nitrogen.

The D-ribose-1-C^{14} had a specific activity of 26.8 millicuries per millimole (manufacturer's data), and was designated CFA, Batch 2.

Analytical Procedures

1) Determination of radioactive isotope.

Radioactive determinations were made in a Packard Tri-Carb Liquid Scintillation Spectrometer, Series 314 E, Packard Instrument Company Inc., La Grange, Illinois. All samples were counted for 10 minutes and no counts were accepted unless the values were at least twice that of background.

Radioactive carbon dioxide was collected from individual eggs in the ethanolamine collecting medium of Jeffay and Alvarez (1961).

Samples of various egg fractions, homogenates, etc., were pipetted directly into the liquid scintillation mixture of Bray (1960).

In the total carbon studies in which egg fractions were broken down with hyamine, an aliquot of the hydrolysate was pipetted into Liqufluor liquid scintillation mixture. Hyamine was obtained from Packard Instrument Company Inc., La Grange, Illinois, and Liqufluor

was obtained from Nuclear Chicago Corporation, Chicago 10, Illinois.

All samples in this study were corrected for color and chemical quenching using the method of Kinnory et al., (1958).

2) Collection of carbon dioxide.

The carbon dioxide collecting device developed by Beakley (1962) was utilized in these studies.

The device consists of twenty four separate egg chambers attached to a manifold. Air from a pump is passed through a 20% KOH carbon dioxide trap, into the manifold, solenoid valves allow the air flow to pass through two egg chambers for 10 minute periods thereby flushing all 24 chambers in a continuous cycle. The carbon dioxide laden air from the egg chambers is collected in individual collection tubes containing 15 ml of ethanol amine solution.

3) Extraction procedures.

a) Extraction of total radioactivity from egg fractions.

Two methods were employed to recover the radioactivity from the egg fractions, they are the total carbon method (modified) of Van Slyke and Folch (1940), and a simple digestion utilizing hydroxide of hyamine 10-X after the method of Herberg (1960).

Upon analysis of the data obtained from both methods it was found that the latter method was by far more efficient, probably due to the fact that the sodium acetate is highly volatile and was lost during the heating of the combustion process and could not be recovered in the

collecting medium employed.

The digestion with hydroxide of hyamine 10-X was carried out by adding 1 ml of hyamine to 0.1 ml of the egg fraction to be analyzed and incubated in the dark at room temperature for 18 hours. The digestions were allowed to proceed in the scintillation vials into which the scintillation counting medium was added directly upon completion of the process.

b) Lipid extraction of infected and uninfected yolk sac.

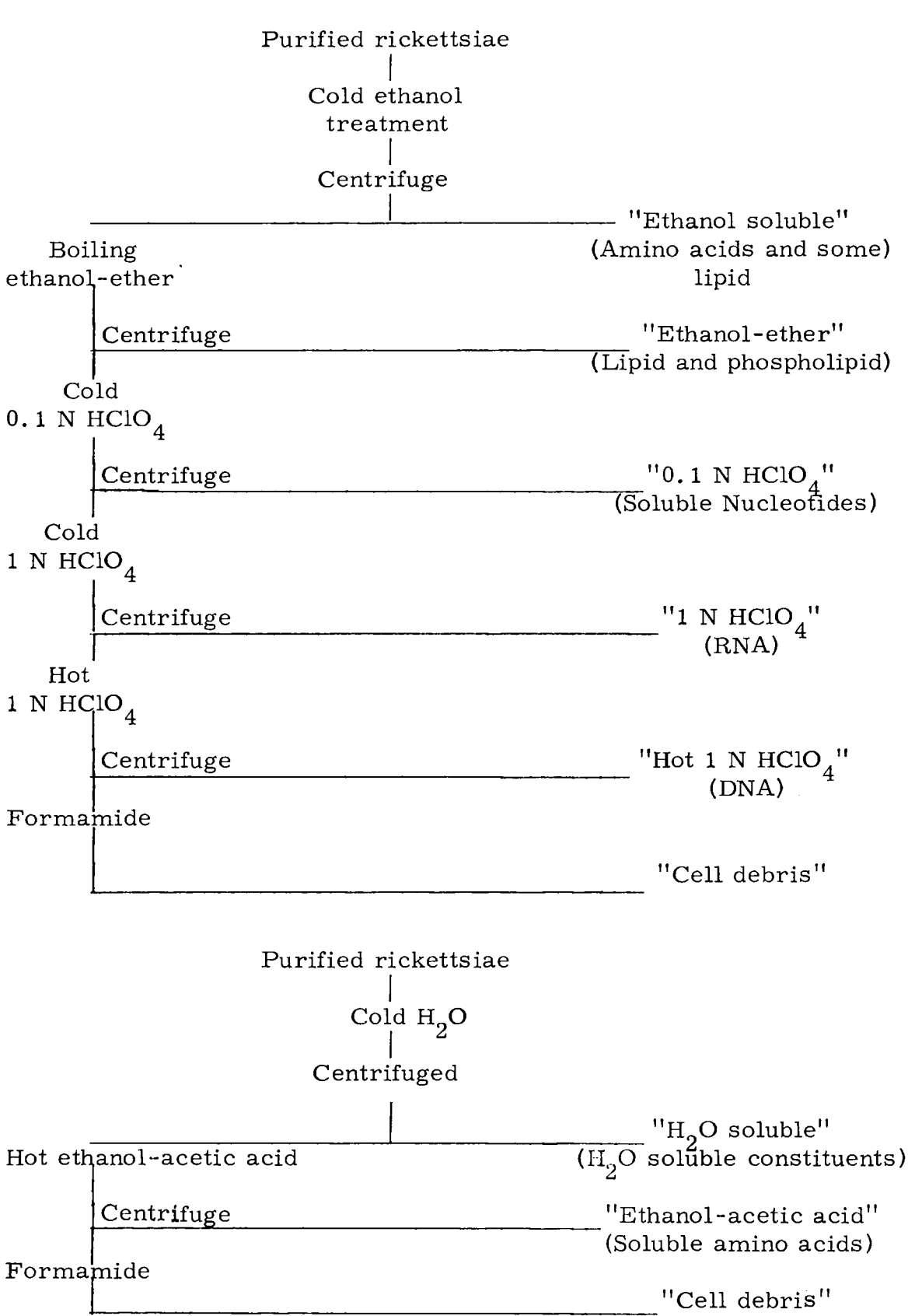
Twelve ml of the yolk sac material to be analyzed was added to 100 ml of ethanol: ether (3:1) solution and magnetically stirred in loosely sealed flasks at 28 C for 18 hours. The cell debris was centrifuged 1 hour at 11,000 r.p.m., the supernatant decanted and the sediment extracted with 10 ml ethanol:ether (3:1) at 70 C for 3 minutes, and centrifuged as above. The latter extraction was repeated until the supernatant appeared clear. The supernatants were combined and stored at 0 C until needed for radioactivity analysis.

c) Extraction of purified rickettsiae.

In both the acetate-U-C^{14} and ribose-1-C^{14} experiments, the purified rickettsial suspensions were divided into three equal parts and treated according to the extraction procedure outlined in Figure 1, and described below.

1) One-third of the rickettsial suspension was treated by a modification of the method of Ogur et al., (1952). The suspension was

Figure 1. Flow-diagrams for the extraction of purified rickettsiae.


Sediment
Supernatant
Purified rickettsiae
Cold ethanol treatment
Centrifuge
"Ethanol soluble" (Amino acids and some) lipid
Boiling ethanol-ether
Centrifuge
"Ethanol-ether" (Lipid and phospholipid)
Cold 0.1 N HClO4
Centrifuge
"0.1 N HClO4" (Soluble Nucleotides)
Cold 1 N HClO4
Centrifuge
"1 N HClO4" (RNA)
Hot 1 N HClO4
Centrifuge
"Hot 1 N HClO4" (DNA)
Formamide
"Cell debris"
Purified rickettsiae
Cold H2O
Centrifuged
"H2O soluble" (H2O soluble constituents)
Hot ethanol-acetic acid
Centrifuge
"Ethanol-acetic acid" (Soluble amino acids)
Formamide
"Cell debris"

rapidly thawed and suspended in 1 ml of 70% ethanol at 4 C for 5 minutes. This was centrifuged at 11,000 r.p.m. for 1 hour at 4 C and the total process repeated 3 times. The supernatant from this extraction was designated the alcohol soluble fraction and stored at -60 C.

The sediment from the preceeding step was extracted by resuspending in 0.1 N $HClO_4$ and 70% ethanol (1:1) for 5 minutes at 4 C and centrifuging at 11,000 r.p.m. for 1 hour. The supernatant was combined with that of the previous step.

The sediment was extracted in ethanol-ethylether (3:1) for 3 minutes at 70 C, and centrifuged as before, this extraction was repeated 3 times. The resulting supernatant was designated the alcohol-ether soluble fraction and stored as before.

The sediment was treated with 0.1N $HClO_4$ at 4 C, centrifuged and the supernatant stored as above.

The sediment from the above was treated with 1N $HClO_4$ at 4 C for 18 hours and centrifuged as above and the supernatant stored.

The sediment was treated with 1N $HClO_4$ at 70 C for 30 minutes, centrifuged, repeated 3 times and the supernatant stored.

The resulting debris was treated for 18 hours with formamide (99%) at room temperature and stored.

2) One third of the rickettsial suspension was treated as follows:

The suspension was rapidly thawed and treated with 3 ml distilled water for 18 hours at 4 C, centrifuged at 11,000 r.p.m. repeated

twice and the supernatant stored at -60 C.

The sediment was treated with 1 ml of 70% ethanol containing 2 drops glacial acetic acid, boiled for 20 minutes, centrifuged, repeated twice, and stored as above.

The sediment was treated with formamide (99%) for 18 hours at room temperature, centrifuged and stored as above.

3) The remaining third of the rickettsial suspension was treated with 1 ml of hyamine for 24 hours at 37 C and stored at -60 C.

4) Determination of specific activity of carbon dioxide.

An efficient method was needed to titrate the amount of carbon dioxide collected in the ethanol amine solution utilized in the sodium acetate-U-C^{14} studies. For this purpose, a modified application of the method of Glenn et al. (1955) was selected.

The titrant employed was sodium amino ethoxide prepared by dissolving 1.25 g. metallic sodium in 50 ml ethanol amine and bringing the solution to 250 ml volume with dimethylformamide. The titrant was standardized with benzoic acid.

The carbon dioxide titrations were carried out by bringing 3 ml of the ethanol amine sample to a 10 ml volume with dimethylformamide. The sample was then titrated with the sodium amino ethoxide solution using three to four grains of thymol blue as an end point indicator. The thymol blue breaks sharply from a pale yellow to a deep blue.

Known samples of calcium carbonate were assayed by this method to determine accuracy and was found to be in the 98-99% confidence range. Ethanol amine blanks were also titrated in the same manner to determine acetic impurities in the solvents, and these values were incorporated in the final figures.

5) Chromatography.

Chromatography was carried out on Whatman No. 1 chromatographic paper obtained from Clico Laboratory Supply Co., Phoenix, Arizona. The paper was cut to a size of approximately 12 x 22 cm. and chromatography performed in one direction only, in tightly sealed glass containers.

a) Solvent.

The solvent employed was the solvent described by Roberts et al., (1963) and was used to separate the lipids in the alcohol-ether fraction of the rickettsiae. The constituents are as follows.

sec butanol ---------- 70 ml

acetic acid ---------- 10 ml

water ---------- 20 ml

b) Reagents.

1) Iodine crystals

Iodine crystals and the chromatograms were placed in a tightly sealed jar after the method of Smith (1960) and the iodine fumes were allowed to penetrate the paper. After three to five minutes, lipid

fractions turned a pale yellow while the paper was uncolored.

2) Phospholipid reagent

Phospholipids were detected with the reagent of Burrows, et al. (1952) which is as follows:

1 gram ammonium molybdate in H_2O	8 vol.
Concentrated HCl	3 vol.
Perchloric acid (12N)	3 vol.
Acetone	86 vol.

The chromatograms were passed through the reagent and dried. The dried chromatograms were exposed to ultra violet light for a period of 10 to 30 minutes. The phospholipid fraction reacts to give a pale blue color which is lasting, the paper becomes dark blue but rapidly fades upon removal from the radiation source.

6) Determination of C^{14} activity of chromatogram fractions.

The lipid and phospholipid fractions identified by chromatography were easily and rapidly assayed for activity utilizing the paper strip counting method of Davidson (1962). The fractions were located on the paper and cut out with a razor, the resulting strips were immersed in Bray's liquid scintillation medium and counted directly.

RESULTS

Respiratory Carbon Dioxide Studies

The respiratory carbon dioxide from individual eggs was collected utilizing the carbon dioxide collection device described earlier. Carbon dioxide was collected at 24 hour intervals in 9 ml of the ethanolamine solution previously mentioned. Three ml of this solution were added to 15 ml of ethanolamine scintillation mixture and the radioactivity determined. The daily accumulative carbon dioxide totals have been plotted against time (Figure 2).

Three ml of the ethanolamine solution were titrated as previously described and the daily specific activity of the eggs injected with sodium acetate-U-C^{14} was determined as counts per minute per μmole of carbon dioxide, and plotted against time (Figure 3).

Upon titration it was found that the average amount of carbon dioxide collected varied less than 0.02/μmole (accumulative) for the three sets of experimental conditions.

It is evident upon examination that the eggs infected with R. typhi demonstrated a marked increase in total activity appearing in the carbon dioxide over that of the controls (Figure 2). The increase in the specific activity of the infected eggs over those of the controls is much more spectacular, approaching 100% on the second day (Figure 3).

FIGURE 2. A comparison of the cumulative radioactivity present in respiratory carbon dioxide from normal eggs, eggs injected with sucrose-P.G., and eggs infected with Rickettsia typhi with sodium acetate-U-C^{14} as a substrate.

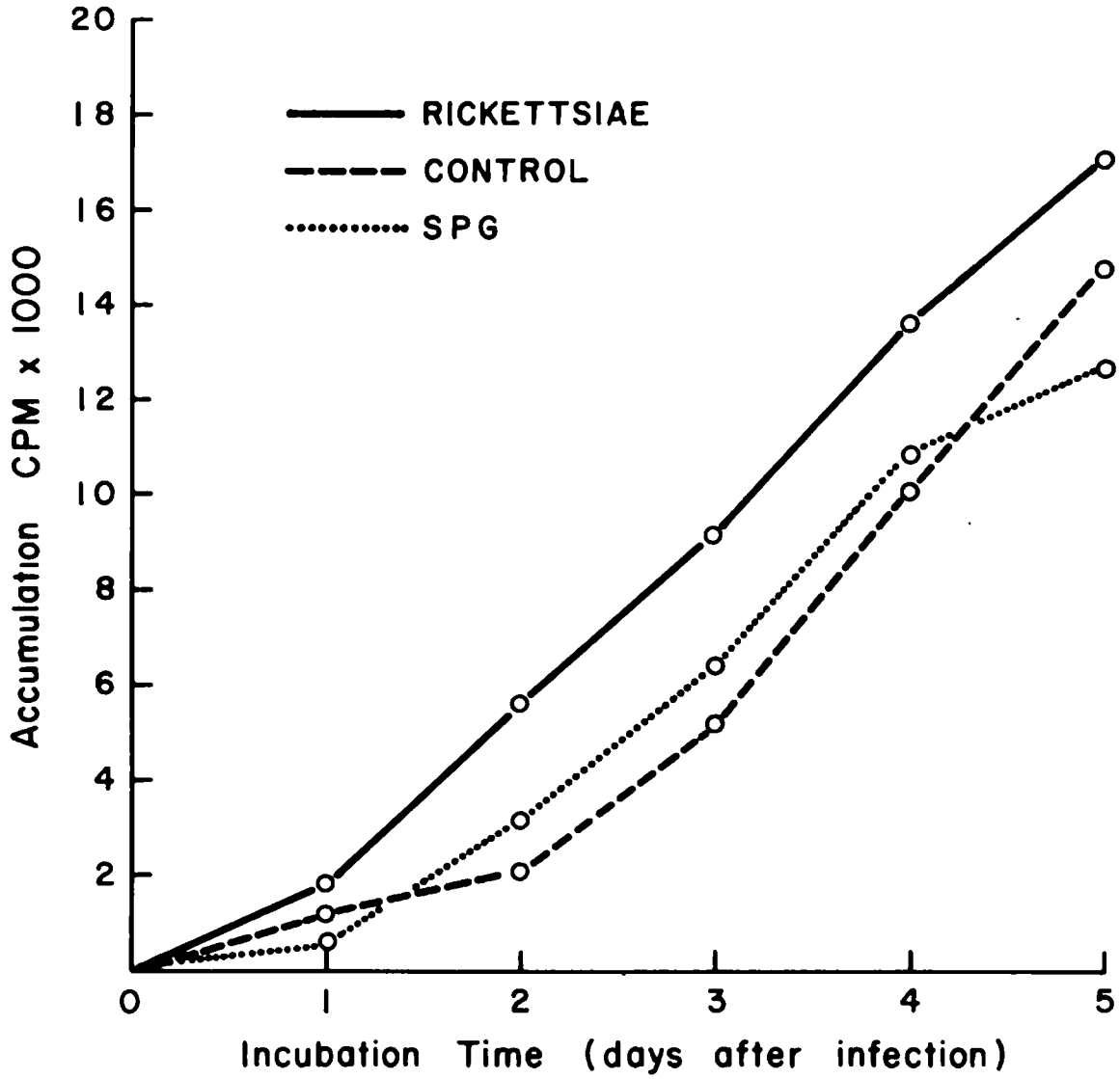
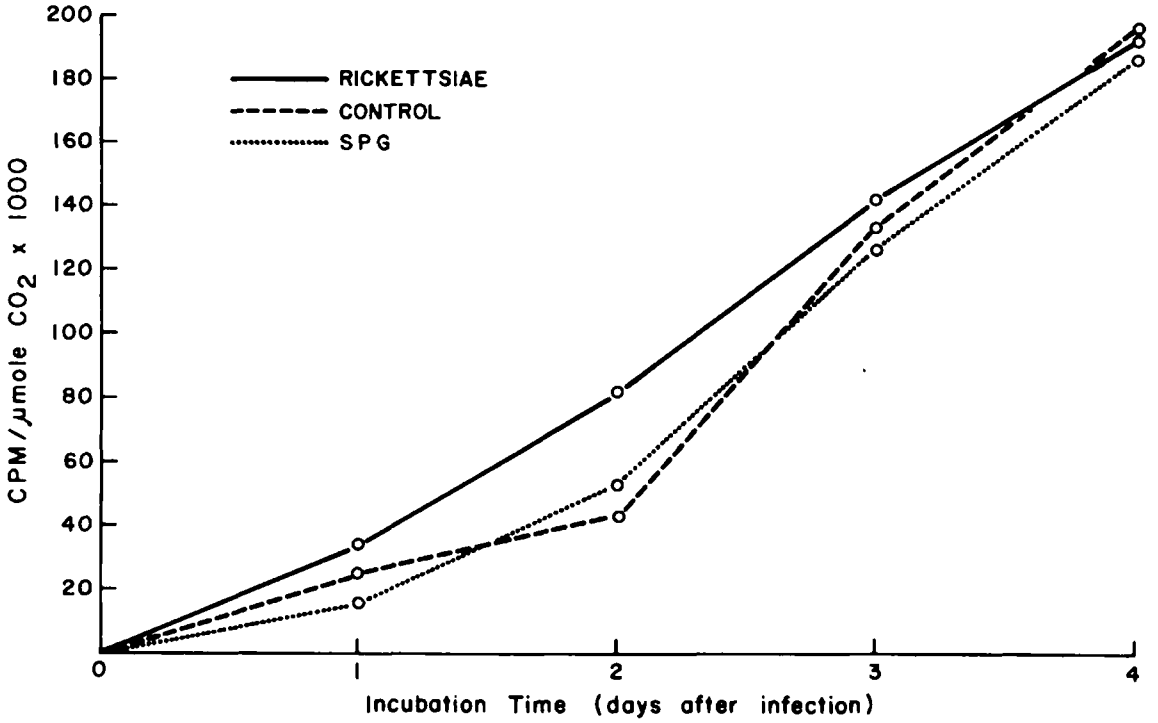

20
18
16
14
12
10
8
6
4
2
0
1
2
3
4
5
RICKETTSIAE
CONTROL
SPG
Accumulation CPM x 1000
Incubation Time (days after infection)

FIGURE 3. A comparison of the cumulative specific activity (cpm/μmole) of respiratory carbon dioxide from normal eggs, eggs injected with sucrose-P.G., and eggs infected with Rickettsia typhi with sodium acetate-U-C^{14} as a substrate.


RICKETTSIAE
CONTROL
SPG
CPM/μmole CO_2 x 1000
200
180
160
140
120
100
80
60
40
20
0
1
2
3
4
Incubation Time (days after infection)

The curves of the uniform control and the sucrose-P. G. control are very nearly similar in both cases.

Distribution of Sodium Acetate-U-C^{14} Derived Radioactivity in Purified Rickettsiae

Table 1 shows the radioactivity present in the various extracts of purified Rickettsiae obtained from the experiments utilizing sodium acetate-U-C^{14} as the substrate. It is evident that the majority of the activity was present in the ethanol-ether soluble fraction and small amounts of activity appeared in the ethanol soluble fraction and the formamide (cell debris) soluble fraction. The amount of activity present in the latter two fractions was found to be so small that no further assay was possible.

Less than 1% of the total activity introduced into the eggs was recovered from the rickettsiae.

Table 2 gives the radioactivity present in the ethanol-ether fraction obtained from the above assay by means of chromatography, which has been previously explained.

It was apparent from this table that the activity was evenly distributed between a lipid and a phospho-lipid fraction with 10% more activity appearing in the lipid fraction.

TABLE 1

ACTIVITY DISTRIBUTIONS OF RICKETTSIAL CELL FRACTIONS

Sodium acetate-U-C^{14} experiment		
Fraction	Total Activity CPM	Distribution Percentages
Ethanol	300	20
Ethanol-ether	1070	72
Cold 0.1 N $HClO_4$	--	0
Cold 1 N $HClO_4$	--	0
Hot 1 N $HClO_4$	--	0
Formamide (cell debris)	121	8
Total rickettsial activity	1491	100

TABLE 2

ACTIVITY DISTRIBUTION IN ETHANOL-ETHER SOLUBLE FRACTION OF RICKETTSIAL CELL EXTRACT AS DETERMINED BY CHROMATOGRAPHY

Sodium acetate-U-C^{14} experiment		
Fraction	Total Activity CPM	Distribution Percentages
Lipid	58	55
Phospho-lipid	48	45

Distribution of Sodium Acetate-U-C^{14} Derived Activity in Lipid Fraction of Yolk Sac

The yolk sac pools of the infected and uninfected egg embryos were extracted separately with ethanol:ether (3:1) as previously described.

Lipid extracted from the infected yolk sac contained 25% more radioactivity in this fraction than that of the controls. When this fraction was chromatographed, it was found that 70% of the radioactivity was in the lipid portion and 30% in the phospholipid portion.

Distribution of Ribose-1-C^{14} Derived Radioactivity in Purified Rickettsiae

Table 3 shows the radioactivity present in the various extracts of purified rickettsiae obtained from the experiments utilizing ribose-1-C^{14} as the substrate. It is evident that no activity appeared in these cells until the cell debris from the earlier extractions was digested with formamide and assayed.

Although this information was pertinent it was deemed necessary to obtain a second portion of these organisms and extract the amino acids. The results of this procedure are listed in Table 4. The activity was distributed between the H_2O, hot ethanol-acetic acid, and formamide soluble fractions. The activity found in these fractions, although significant, was found to be too small for further assay, and will be discussed later.

TABLE 3

ACTIVITY DISTRIBUTIONS IN RICKETTSIAL CELL FRACTIONS

Fraction	Ribose-1-C^{14} experiment: Total Activity CPM	Distribution Percentages
Ethanol	--	0
Ethanol-ether	--	0
Cold 0.1 N $HClO_4$	--	0
Cold 1 N $HClO_4$	--	0
Hot 1 N $HClO_4$	--	0
Formamide (cell debris)	662	100

TABLE 4

ACTIVITY DISTRIBUTIONS UPON EXTRACTION OF RICKETTSIAL CELLS FOR AMINO ACIDS

Fraction	Ribose-1-C^{14} experiment: Total Activity CPM	Distribution Percentages
H_2O	64	8
Hot ethanol-acetic acid	580	73
Formamide (cell debris)	154	19

Comparison of Sodium Acetate-U-C^{14} and Ribose-1-C^{14} Radioactivity Distribution in Purified Rickettsiae

Table 5 was compiled in an attempt to demonstrate the relative distribution comparisons between rickettsial extracts when sodium acetate-U-C^{14} and ribose-1-C^{14} were used as substrates.

It is evident from these data that there is a wide spread in the radioactivity between the fractions derived from the two substrates. In the organisms supplied sodium acetate-U-C^{14}, the majority of the activity appears in the ethanol-ether fraction while in the organisms supplied ribose-1-C^{14}, the majority of the activity appeared in the ethanol-acetic acid fraction. In both cases there is a lack of activity in the $HClO_4$ soluble fractions.

TABLE 5

COMPARISON OF ACTIVITY DISTRIBUTIONS IN RICKETTSIAL FRACTIONS BY PERCENTAGES

Fraction	ISOTOPE POOL	
	Sodium Acetate-U-C^{14}	Ribose-1-C^{14}
Ethanol	20	0
Ethanol-ether	72	0
Cold 0.1 N $HClO_4$	0	0
Cold 1 N $HClO_4$	0	0
Hot 1 N $HClO_4$	0	0
H_2O	0	8
Formamide (cell debris)	8	19
Ethanol-acetic acid	0	73

DISCUSSION

The approach to the study of rickettsial metabolism and its effect on the host, in relation to the substrates introduced, is at best a difficult one, and the data are necessarily difficult to analyze due to the many complex contributing factors.

This study attempted to approach this problem by two methods.

a) The assay of the radioactive byproducts of metabolism, specifically carbon dioxide, in an attempt to determine the effect of the parasite upon the host, and the host's metabolism of the substrate introduced.

b) Assay of radioactivity in the rickettsial fractions to determine in what way the parasite incorporates the label introduced through the host.

Upon analysis of the data collected in Figure 2 it is readily apparent that the infected eggs demonstrated a substantially higher radioactivity in the respired carbon dioxide. This may be accounted for in one of two ways. Either more carbon dioxide was respired by the infected eggs or more of the label was being introduced into this carbon dioxide.

If one considers that the data obtained from the carbon dioxide titrations indicate the amount of carbon dioxide is substantially the

same for the infected and uninfected eggs, and that the specific activity data (Figure 3) indicate that more radioactivity per unit of carbon dioxide is incorporated in the infected eggs; then it is obvious that more of the label from the substrate introduced is incorporated in the carbon dioxide respired by the infected eggs.

When one considers the above data along with the previously mentioned fact that a substantially larger amount of radioactivity (25%) appeared in the lipid fraction of infected yolk sac over that of the controls, it is apparent that infection with R. typhi has a marked effect on the eggs metabolism, and a certain pattern of this effect begins to emerge.

Since it was demonstrated by Berg (1956; A, B, and C) that acetate is rapidly converted to acetyl coenzyme A (Acetyl CoA) in the presence of ATP, Mg^{+2} ions and CoA, it is evident that acetate in this form lies at the intersection of the trunk lines of glycolysis, the tricarboxylic acid (TCA) cycle, lipid metabolism, and is directly involved in the glyoxylate cycle (Kornberg and Krebs, 1957).

Kornberg (1958) demonstrated that uniformly labeled acetate when fed to cells caused a rapid appearance of label in all the intermediates of the TCA cycle, and many studies upon lipid synthesis, such as those of Smedley-Maclean and Hoffert (1926), utilizing this substrate show a continuous turnover of label in the lipid pool.

In view of the fact the data showed a higher specific activity of the carbon dioxide evolved from the infected eggs, it cannot be assumed that the tricarboxylic acid cycle nor the glyoxylate cycle alone are responsible for this rise. If this were so the specific activity would be equal for all the groups.

It appeared that activity is being cycled into this system in some manner, in this way more of the carbon dioxide would be labeled without the amount being increased.

The evidence presented that there is an apparent acceleration of lipid synthesis in infected eggs could very well explain the increase in the specific activity in the carbon dioxide of these eggs. The oxidation of the fatty acids and the constant recycling of these carbon sources into the tricarboxylic acid cycle seems the most reasonable method for added activity being incorporated into the carbon dioxide.

This evidence seemed more conclusive when one considered the fact that the majority of the activity found in the purified rickettsiae was found in the lipid and phospholipid fractions. This agrees with the in vitro studies of Bovarnick (1960) when she found all of the activity of acetate-1-C^{14} fed to resting cells was found in the lipid fraction. The fact that some activity was also found in the protein fraction of the rickettsiae used in this study differs from her data. This was undoubtedly due to the difference in the fact that she studied rickettsiae in a resting state and not in intracellular metabolism.

It must also be considered that the dilution of radioactivity in respired carbon dioxide caused by the sucrose-P.G. suspending medium observed by Beakley (1962) and Swack (1965) was not a factor in this study. This would tend to support the lipid hypothesis offered above, because it does not seem reasonable that the sucrose or the glutamate would enter into lipid condensation preferentially or at the same rate over available acetate.

The various condensations of acetate and reactions of acetyl phosphate can of course play a role in these reactions but it would seem to this author that if these played any major role other than lipid synthesis or cycling into the tricarboxylic acid cycle, that there should then be a higher percentage of activity appearing in the protein fraction of yolk sac and rickettsiae.

The effect of R. typhi upon the metabolism of ribose-1-C^{14} in this study is much more difficult to analyze, but one fact is immediately apparent, the ribose is not being incorporated into the nucleic acids of the rickettsiae (Table 3).

The rickettsiae do incorporate a certain amount of the label in the soluble and insoluble protein fractions, and possibly unidentified substances in the water soluble and cell debris fractions. These observations tend to indicate that the ribose is undergoing an intermediate metabolism in the egg.

It has been reported that ribose is easily metabolized by means of the pentose pathway and eventually cycled into the tricarboxylic acid cycle. The substance "active" glycolaldehyde is formed in this process, but this compound does not incorporate the labeled carbon.

The pentose pathway can explain the presence of activity in the amino acids of the rickettsiae. Although the active carbon is respired as CO_2 after α ketoglutarate in the tricarboxylic acid cycle, condensations of citrate, isocitrate, and α ketoglutarate could lead to the incorporation of activity in the various amino acids and their intermediates.

SUMMARY

It has been demonstrated that infection with Rickettsia typhi causes an apparent shift of host metabolism toward increased synthesis of lipid. Carbon dioxide recovered from infected eggs supplied sodium acetate-U-C^{14} had a higher specific activity than that of the controls, and infected yolk sac exhibited a substantially higher activity in the lipid fraction.

Rickettsiae extracted and purified from the host system injected with acetate-U-C^{14} were fractionated and the majority of the activity appeared in the lipid and phospholipid fractions.

Rickettsiae recovered from the host system previously injected with ribose-1-C^{14} exhibited a lack of activity in the RNA and DNA fractions, although these organisms did contain small amounts of activity in the various amino acids and unidentified cell components.

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