

UNCLASSIFIED

AD NUMBER
AD448485
NEW LIMITATION CHANGE
TO Approved for public release, distribution unlimited
FROM Distribution authorized to U.S. Gov't. agencies and their contractors; Administrative/Operational Use; AUG 1964. Other requests shall be referred to US Army Biological Labs., Attn: Technical Releases Branch/TID, Fort Detrick, Frederick, MD 21701.
AUTHORITY
US Army Biological Defense Research Lab ltr dtd 27 Sep 1971

THIS PAGE IS UNCLASSIFIED

UNCLASSIFIED

AD 4 4 8 4 8 5

DEFENSE DOCUMENTATION CENTER

FOR

SCIENTIFIC AND TECHNICAL INFORMATION

CAMERON STATION, ALEXANDRIA, VIRGINIA



UNCLASSIFIED

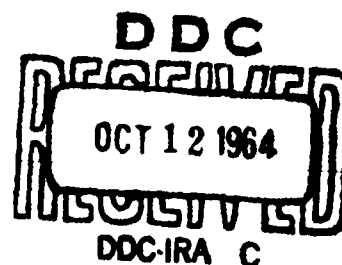
NOTICE: When government or other drawings, specifications or other data are used for any purpose other than in connection with a definitely related government procurement operation, the U. S. Government thereby incurs no responsibility, nor any obligation whatsoever; and the fact that the Government may have formulated, furnished, or in any way supplied the said drawings, specifications, or other data is not to be regarded by implication or otherwise as in any manner licensing the holder or any other person or corporation, or conveying any rights or permission to manufacture, use or sell any patented invention that may in any way be related thereto.

443485

TECHNICAL MANUSCRIPT 155

**RICKETTSIAL GROWTH
IN SUSPENSION CELL CULTURES
IN SERUM-FREE AND DEFINED MEDIA**

AUGUST 1964



**UNITED STATES ARMY
BIOLOGICAL LABORATORIES
FORT DETRICK**

U.S. ARMY BIOLOGICAL LABORATORIES
Fort Detrick, Frederick, Maryland

TECHNICAL MANUSCRIPT 155

RICKETTSIAL GROWTH IN SUSPENSION CELL CULTURES
IN SERUM-FREE AND DEFINED MEDIA

Henry R. Tribble, Jr.

Horace B. Rees, Jr.

Henry J. Hearn, Jr.

Medical Bacteriology Division
and
Virus and Rickettsia Division
DIRECTOR OF BIOLOGICAL RESEARCH

Project 1C522301A05901

August 1964

This publication or any portion thereof may not be reproduced without specific authorization from the Commanding Officer, U. S. Army Biological Laboratories, ATTN: Technical Releases Branch, Technical Information Division, Fort Detrick, Frederick, Maryland. 21701. However, DDC is authorized to reproduce the publication for U. S. Government purposes.

The information in this publication has not been cleared for release to the public.

DDC AVAILABILITY NOTICE

Qualified requestors may obtain copies of this publication directly from DDC.

Foreign announcement and dissemination of this publication by DDC is limited.

ABSTRACT

Rickettsia typhi and Rickettsia rickettsii were propagated in cultures of cat kidney and L cell lines that were grown in a serum-free suspension system. One growth medium (LAH) consisted of lactalbumin hydrolyzate and glutamine as nitrogen sources, glucose and pyruvate as carbon sources, vitamins, salts, and additives such as Methocel, insulin, and phenol red. A second and third medium consisted of essential amino acids (EAA) with or without nonessential amino acids (NEAA), respectively as nitrogen sources, salts, carbon sources, vitamins, and the additives, Methocel, insulin and phenol red. LAH supplemented with 10 per cent calf serum was used as a control.

The two cell lines were carried as stock cultures in serum-free suspensions at least a month prior to the experiments. The studies were conducted with 100-ml serum bottles (30 ml medium per bottle) and were agitated on a New Brunswick shaker at 130 rpm at 35°C. A maximal yield of $10^{6.8}$ yolk sac (YS) LD₅₀ of R. typhi occurred at 7 days in cat kidney cells grown in EAA; titers were slightly less in LAH and considerably less in NEAA cell cultures.

In contrast, maximal yields of $10^{5.8}$ YSLD₅₀ of R. rickettsii occurred at 4 days in L cells grown in NEAA; slightly lower titers were found in EAA. In LAH only minimal amounts of rickettsiae were found. Titers obtained with cultures infected with R. typhi suggested cyclic rickettsial growth.

These data, obtained in serum-free and chemically defined media, demonstrate the importance of nutritional environments for maximum survival, penetration, and proliferation of rickettsial species and suggest a new approach to studying rickettsial metabolism.

I. INTRODUCTION

Studies that define the nutritional requirements for rickettsial multiplication in animal cell cultures have received increasing attention during the last two decades. A limiting factor in these studies has been the need for a serum for the growth of animal cell cultures. A system in which experiments could be conducted with serum-free media or with chemically defined media would be desirable. Several such investigations have been reported^{1,2} at meetings and in the literature in recent years. This paper presents the results of preliminary studies on the growth of Rickettsia rickettsii and Rickettsia typhi in suspended cell cultures employing serum-free and chemically defined media.

II. MATERIALS AND METHODS

The media employed were as follows: (a) A serum-free lactalbumin hydrolyzate medium designated as LAH (Table I) which contained lactalbumin hydrolyzate and glutamine as nitrogen sources, glucose and pyruvate as carbon sources, vitamins, salts, plus such additives as Methocel and phenol red; (b) a defined medium designated as EAA (Table II), which contained 13 essential amino acids, carbon sources, salts, vitamins and Methocel and phenol red; and (c) the defined medium plus six amino acids found nonessential for cell growth, and hereafter referred to as NEAA. In studies with R. typhi, a fourth type of medium, LAH supplemented with 10 per cent calf serum, was employed. The medium with serum was added to serum-free cat kidney cells grown just prior to infection. This combination served as an initial positive control for the growth of rickettsiae.

Animal cell cultures, suspended in 30 ml of media contained in 100-ml serum bottles were incubated at 34°C in a New Brunswick gyrotory shaker rotating at 130 rpm. Both cat kidney and L cell lines were derived from stocks maintained in either the LAH medium or the defined medium for at least a month before infection with rickettsiae.

The Bitterroot strain of R. rickettsii and the Wilmington strain of R. typhi (R. mooseri) were prepared as inocula from infected yolk sacs of embryonated eggs by a modified, molar potassium chloride method of Ormsbee.⁴ After the final centrifugation, rickettsiae were suspended in Bovarnick's⁵ sucrose-phosphate-glutamate containing 0.6 per cent bovine albumin (Armour's Fraction V) and stored at -65°C until the mixtures were thawed and employed as inocula.

TABLE I. LACTALBUMIN HYDROLYZATE MEDIUM FOR SUSPENDED CELL GROWTH

Component	Concentration, mg/l	Component	Concentration, mg/l
Nitrogen Sources:		Salts:	
Lactalbumin hydrolyzate	2500	NaCl	7400
L-Glutamine	300	KCl	400
Vitamins:		NaH ₂ PO ₄ ·H ₂ O	100
D-biotin	1.0	NaHCO ₃	300
Choline·Cl	1.0	CaCl ₂ ·2H ₂ O	265
Folic Acid	1.0	MgCl ₂ ·6H ₂ O	275
Niacinamide	1.0	Carbon Sources:	
Ca pantothenate	2.0	Glucose	1000
Pyridoxal·HCl	1.0	Sodium pyruvate	110
Thiamine·HCl	1.0	Antibiotics, etc.:	
i-inositol	1.0	Methocel 15 cps	1000
Riboflavin	0.1	Phenol red	10 mg/l
B ₁₂	0.002		

TABLE II. CHEMICALLY DEFINED MEDIUM FOR SUSPENDED CELL GROWTH

Component	Concentration, mg/l	Component	Concentration, mg/l
Amino Acids (essential):		Salts:	
L-Arginine·HCl	100	NaCl	7400
L-Cysteine·HCl	75	KCl	400
L-Histidine·HCl	60	NaH ₂ PO ₄ ·H ₂ O	100
L-Isoleucine	150	NaHCO ₃	500
L-Leucine	300	CaCl ₂ ·2H ₂ O	265
L-Lysine	300	MgCl ₂ ·6H ₂ O	275
L-Methionine	60	Carbon Sources:	
L-Phenylalanine	120	Glucose	1000
L-Threonine	135	Sodium pyruvate	110
L-Tryptophan	60	Vitamins:	
L-Tyrosine	120	D-biotin	1.0
L-Valine	150	Choline·Cl	1.0
L-Glutamine	450	Folic Acid	1.0
Amino Acids (nonessential):		Niacinamide	1.0
Glycine	60	Ca pantothenate	2.0
L-Alanine	120	Pyridoxal·HCl	1.0
L-Serine	150	Thiamine·HCl	1.0
L-Cystine·HCl	75	i-inositol	1.0
L-Aspartic Acid	270	Riboflavin	0.1
L-Glutamic Acid	315	B ₁₂	0.002
L-Proline	115		
Antibiotics, etc.:			
Methocel 15 cps	1000		
Phenol red	10 mg/l		

Cell cultures with populations from 5×10^5 to 1.5×10^6 cells per ml were inoculated with these partially purified rickettsiae. The mixture was incubated for 45 minutes on the rotary shaker-incubator and centrifuged to sediment the cells. Supernatant medium and unadsorbed rickettsiae were removed by decanting, and the remaining cells resuspended in fresh medium. These resuspended cells were then sampled for a rickettsial assay and a cell count. The cultures were separated from spent medium by centrifuging every other day and resuspended in fresh medium. Additional culture samples were obtained daily, diluted 1:1 with normal calf serum, sealed in glass ampoules, and stored at -65°C until assayed for rickettsial titer in embryonated eggs. Viable tissue cell counts were made using the 0.5 per cent trypan blue method.

III. RESULTS AND DISCUSSION

To assay rickettsial infectivity, tenfold dilutions of each sample were inoculated into the yolk sac of 7-day embryonated eggs. After incubating the eggs at 35°C in a commercial incubator for 11 days, embryo deaths were determined daily by candling. Embryo deaths occurring in the typical pattern for rickettsial infections were employed to calculate the LD_{50} titer per ml by the method of Reed and Muench.

The stabilities of rickettsial infectivity at 34°C in a shaker-incubator were determined first in LAH and defined media without tissue cells. Ten per cent skimmed milk was included also for comparison. *R. typhi* and *R. rickettsii* showed similar losses of infectivity (Figure 1). Each lost one or more logarithms of infectivity after four hours at 34°C . Other data indicated little or no infectivity remained after 24 hours of incubation. These findings suggested that rickettsial infectivity found after 24 hours must have been a result of cell-rickettsial association.

When cells of the cat kidney line were infected with *R. typhi* (Figure 2), cultures grown either in LAH or LAH plus 10 per cent calf serum appeared to produce cyclic yields of rickettsiae. The highest yields of rickettsiae were about 10^5 YSLD_{50} per ml and occurred in the LAH medium on the 4th, 14th, and 20th day after inoculation, as shown by the open circles in the figure. The highest yield found for the cells grown in the medium with serum was $10^{4.5}$ infectious units and occurred on the ninth day of the infection. Neither rickettsial yields nor the cell proliferation appeared to be enhanced by the addition of 10 per cent calf serum to the LAH.

The number of cat kidney cells remained static for the first six days of rickettsial infection, then, a precipitous drop in the number occurred on the Day 7. This was followed by a slow decline in cell numbers until complete cell lysis occurred on Day 15 in the medium with serum, or on Day 20

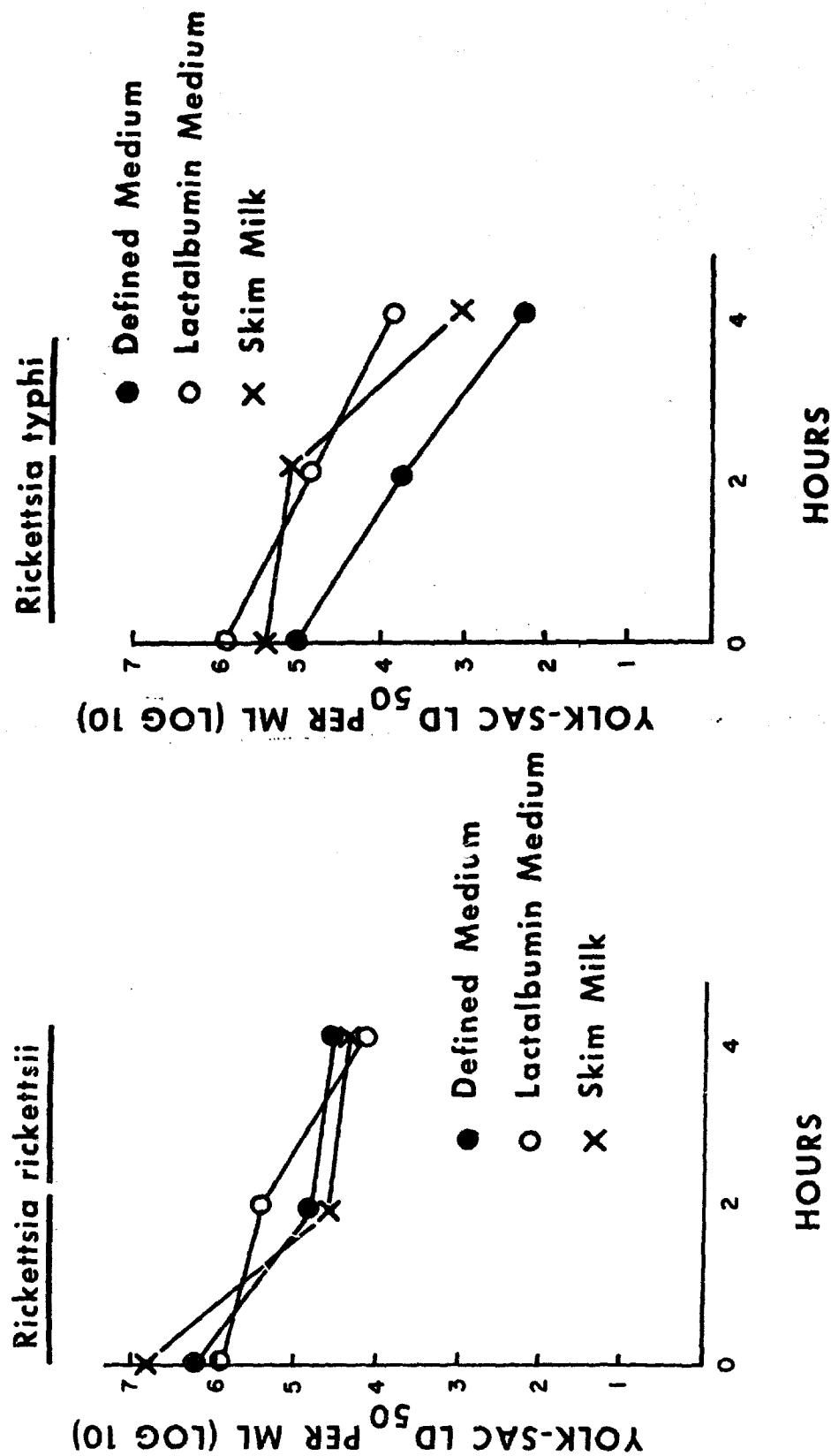


Figure 1. Stability of *Rickettsia* in Cell-Free Tissue Culture Media in a Gyrotory Shaker at 34°C.

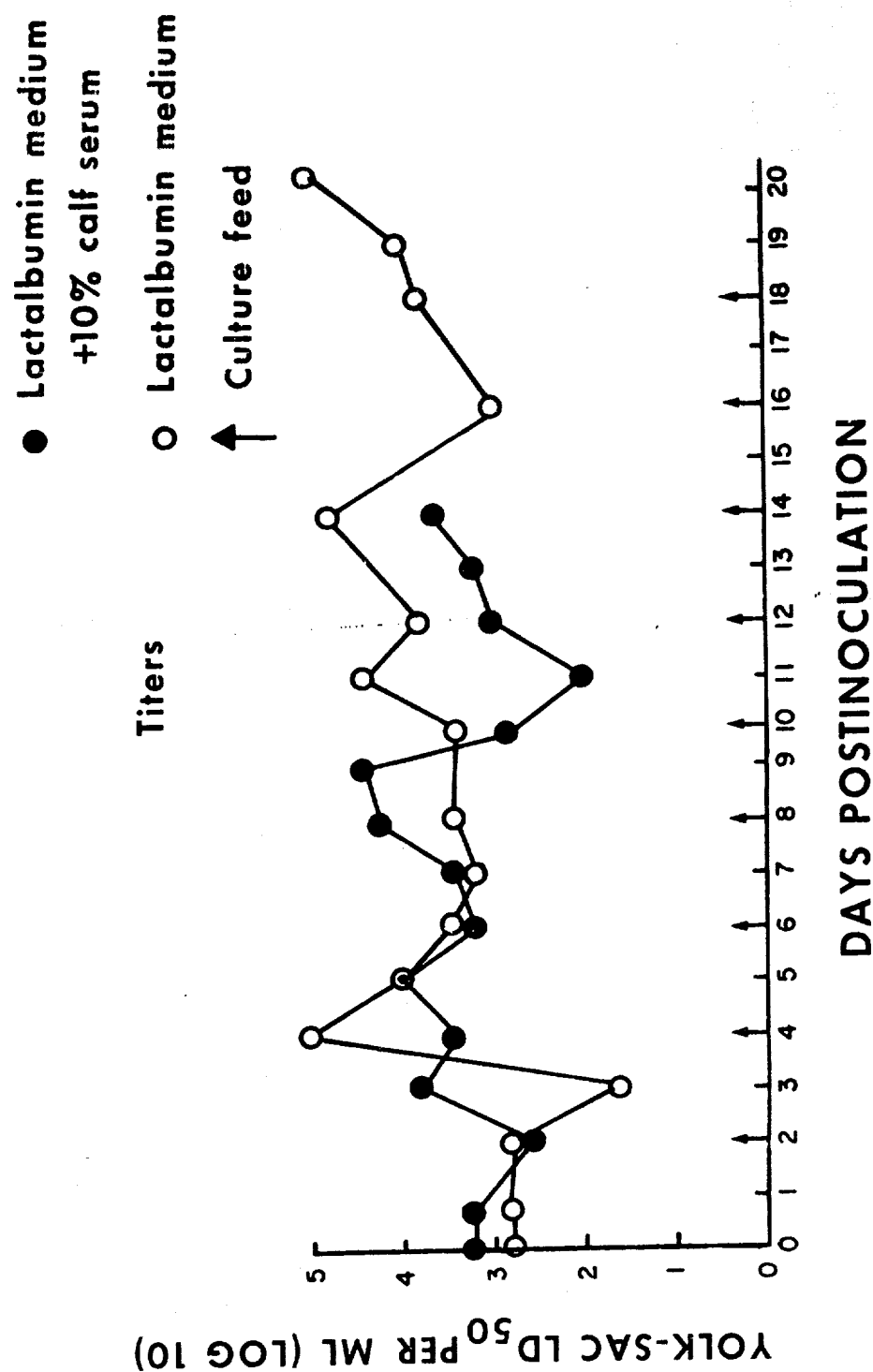


Figure 2. Growth of *R. typhi* in Cat Kidney Cell Line Suspension Culture.

in the LAH medium. Increases in the rates of cell lysis were generally associated with increased rickettsial yields.

When growth curves of *R. typhi* were determined in cultures of the cat kidney line propagated in EAA and NEAA media (Figure 3), the tissue cells grown in EAA yielded greater amounts of rickettsiae than did those cells grown in the same medium supplemented with nonessential amino acids. This is shown by the closed circles in the figure, which depict maximal rickettsial yields of $10^{5.8}$ YSLD₅₀ per ml in the defined medium, compared to $10^{5.3}$ YSLD₅₀ per ml for cells in the defined plus NEAA medium (open circles). A culture life of 8 days was not sufficient to confirm the cyclic yields of rickettsiae observed in the previous experiment. However, a comparison of the rickettsial yields with the number of cells in cultures again suggested that maximal infectivity was closely associated with cell lysis.

With *R. rickettsii* in L cell cultures the beneficial effect of incorporating the nonessential acids was observed (Figure 4). Cells infected with spotted fever rickettsiae and grown in NEAA produced a maximal yield of $10^{6.3}$ YSLD₅₀ per ml on the sixth day after infection, shown as the solid circles in the figure. By comparison, cells in the defined EAA medium produced only $10^{5.5}$ YSLD₅₀ per ml. In marked contrast, infected cells suspended in LAH medium grew well but failed to support proliferation of the rickettsiae.

In summary, a new approach to studying rickettsial metabolism has been demonstrated in cells propagated in serum-free and chemically defined environments. Preliminary data have suggested rather striking differences among media and rickettsial species for maximal survival, penetration, and proliferation.

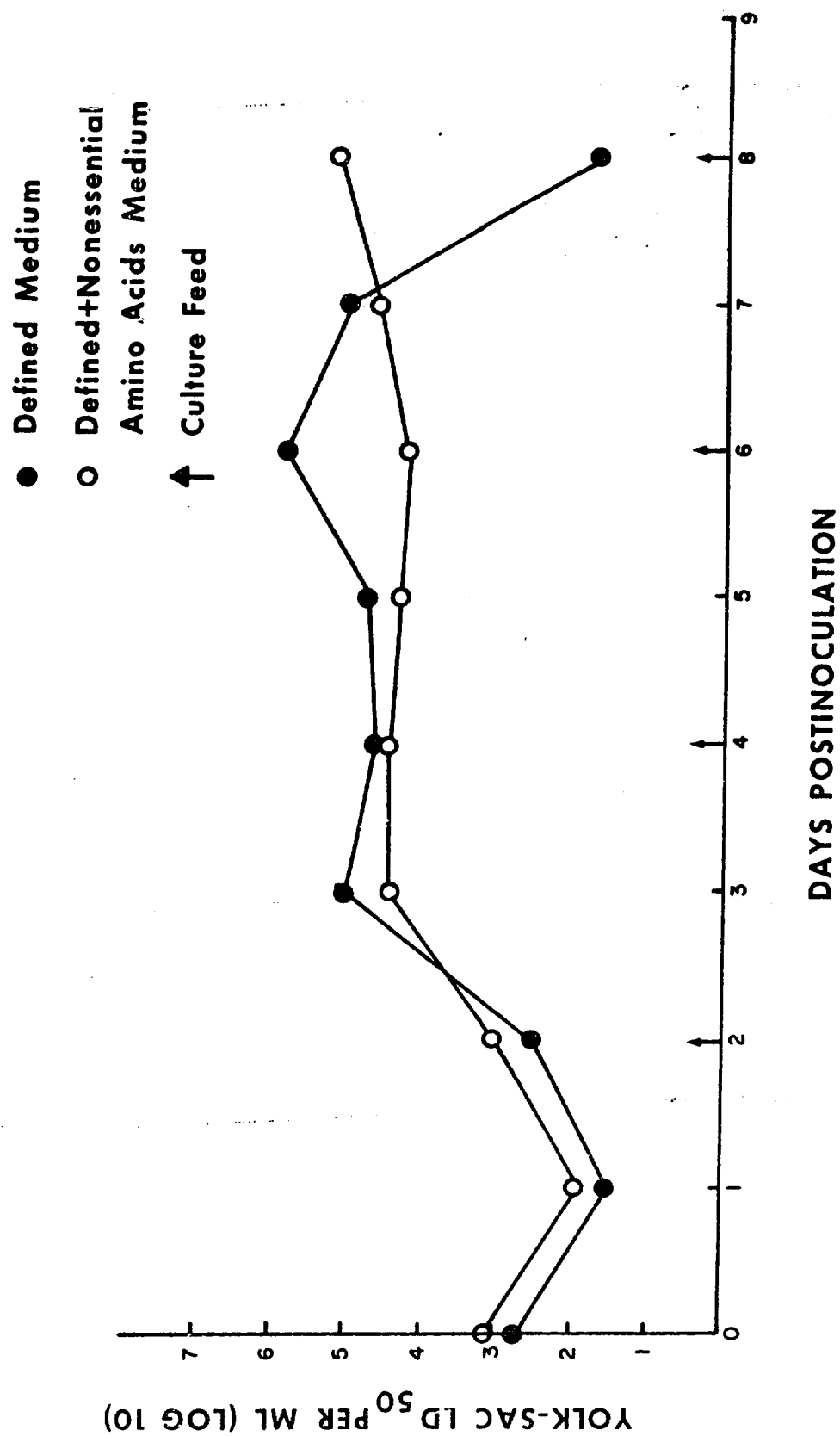


Figure 3. Growth of *R. typhi* in Cat Kidney Cells.

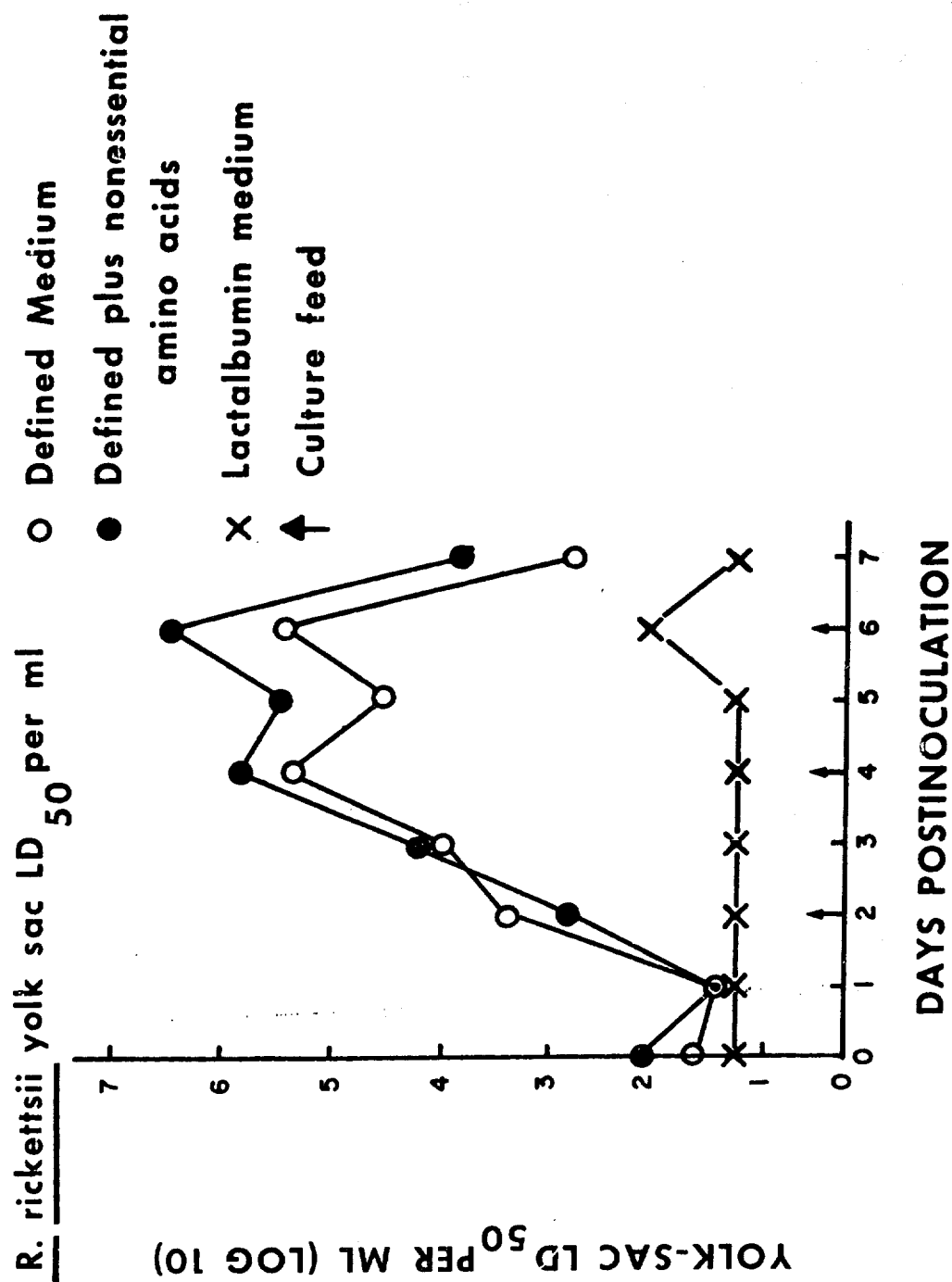


Figure 4. Growth of R. rickettsii in L Cell Suspension Culture.

LITERATURE CITED

1. Tribble, H.R., Jr., and Higuchi, K. "Studies on the nutrition and metabolism of animal cells in serum-free media: II. Cultivation of cells in suspension," J. Infect. Diseases 112:221-225, 1963.
2. Nagle, S.C., Jr.; Tribble, H.R., Jr.; Anderson, R.E.; and Gary, N.D. "A chemically defined medium for growth of animal cells in suspension," Proc. Soc. Exptl. Biol. Med. 112:340-344, 1963.
3. Bozeman, F.M.; Hopps, H.E.; Danauskas, J.X.; Jackson, E.B.; and Smadel, J.E. "Study on the growth of rickettsiae: I. A tissue culture system for quantitative estimates of Rickettsia tsutsugamushi," J. Immunol. 76:475, 1956.
4. Ormsbee, R.A. "A method of purifying Coxiella burneti and other pathogenic rickettsiae," J. Immunol. 88:100-108, 1962.
5. Bovarnick, M.R.; Miller, J.C.; and Snyder, J.C. "The influence of certain salts, amino acids, sugars, and proteins on the stability of rickettsiae," J. Bacteriol. 59:509-522, 1950.