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Nutritional Studies of *Rickettsia quintana*: Nature of the Hematin Requirement

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Rickettsia quintana grew in a liquid medium consisting of a brain-heart infusion base supplemented with starch and hematin. The growth requirement for hematin could not be substituted by compounds of known catalytic activity for H_2O_2, viz., catalase, potassium pyruvate, or charcoal, or by the reducing compounds sodium sulfite and sodium thioglycollate. *R. quintana* was catalase-negative, but no H_2O_2 production could be demonstrated by the catalase-aminotriazole technique. A minimum inoculum giving 10^5 cells/ml was required to initiate growth. The generation time at 33 C was 10 hr. The temperature range for growth was 28 to 37 C. Growth was enhanced when succinate or glutamate was added as energy source.

Previous studies from this laboratory indicated the ability of crystalline hemoglobin or hematin to substitute for a red blood cell lysate in the growth of *Rickettsia quintana* (16). Lascelles, in discussing the nature of the hematin requirement for the growth of various bacteria, pointed out a divergence in the nature of this requirement among these organisms (12). *Haemophilus influenzae* has a requirement in the range of 0.05 to 0.3 μg/ml, and only certain related porphyrins can substitute. However, in certain other organisms, such as *Pasteurella pestis*, *Bordetella pertussis*, or catalase-negative strains of *Mycobacterium tuberculosis*, the hematin requirement is 10 to 100 times higher. In this latter case, a variety of compounds can substitute for hematin, including catalase, potassium pyruvate and other keto-acids, charcoal, and certain sulfhydryl compounds, all of which possess in common the ability to degrade H_2O_2. In this study we have investigated the nature of the hematin requirement for *R. quintana* growing in a liquid medium. In addition, certain other aspects of growth in our liquid medium were examined, including growth temperature range, generation time, and threshold inoculum size.

MATERIALS AND METHODS

The basal liquid medium contained brain-heart infusion (BBL), 3.7%; Thiotone (BBL), 0.5%; soluble starch, 0.1%; and succinic acid, 17 mM, unless otherwise specified. The *p*H before autoclaving was 7.4. The complete medium, which was employed for stock culture maintenance and in certain experiments, was obtained by the addition of sterile hematin (20 μg/ml) to the previously autoclaved basal medium.

When used, charcoal, sodium thioglycolate, and sodium sulfite were sterilized in the basal medium by autoclaving (121 C for 15 min). Hematin, catalase, and pyruvate were sterilized separately by membrane filtration (Millipore GS, 0.22 μm, with a glass fiber prefilter).

The hematin stock solution was prepared from hemin (recrystallized, Nutritional Biochemicals Corp.) as previously described (16) with the added refinement that, because a small insoluble residue remains, the amount of hematin in true solution was determined spectrophotometrically (E^{572}_{mM} = 5.5, at *p*H 7.0) and then diluted to a stock concentration of 100 μg/ml. Catalase (sterile, beef liver, Nutritional Biochemicals Corp.) was standardized by the spectrophotometric procedure of Beers and Sizer (1).

The Fuller strain of *R. quintana*, obtained from J. W. Vinson (19), was used throughout this study. The stock culture was grown in the complete medium, and transfers were made at 48-hr intervals by inoculating 0.1 to 0.5 ml of a logarithmic phase culture to 5 ml of new medium. At approximately 1-month intervals a new culture was started from a frozen preparation for a new series of transfers. When used as inoculum for an experiment, the organisms, in the late logarithmic phase, were centrifuged (5,000 × *g*, 15 min), the supernatant fluid was removed, and the cells were suspended to the same volume in the basal medium. The inoculum (0.5 ml) was then added to the test medium (4.5 ml) to give a starting absorbance of 0.05 to 0.10.

Because of the obligately aerobic nature of *R. quintana*, the cultures (5 ml) were incubated either

as a thin layer in side arm Erlenmeyer flasks (Nephelo culture flasks, 300 ml, Bellco) without shaking, or in screw cap culture tubes, 16 by 125 mm, in a roller drum (2 rev/min). With the exception of the temperature range experiments where temperatures other than 33 C were used, all cultures were incubated at 33 C in a water-jacketed CO_2 incubator at 95% air:5% CO_2. For the other temperatures, a conventional bacteriological incubator was employed. The 5% CO_2 requirement was met in these cases by incubating the side arm flasks in a 5-gal size Case jar, the CO_2 being produced by adding acid to a measured quantity of sodium bicarbonate.

Growth was measured by turbidity with a Spectronic 20 spectrophotometer at 725 nm wavelength. Viable cell counts were made on commercial blood agar plates (BBL) by using the technique of Miles and Misra (14). Colonies were counted under low-power magnification after 7 days of incubation.

Catalase activity was determined in a Warburg respirometer. A washed cell suspension of *Escherichia coli* B used as a positive control was incubated for 30 min at 37 C to reduce endogenous respiration before being used in the respirometer. The *E. coli* and *R. quintana* cell suspensions were adjusted to an absorbance of 3.0 (725 nm, 1 cm) in 1.5 ml of 0.05 M sodium phosphate buffer, *p*H 7.0. Hydrogen peroxide (0.5 ml, 0.04 M) was placed in the side arm. The flasks were shaken at 70 cycles/min at 12 C. Both cultures were incubated for 15 min before the peroxide was tipped to determine whether any gaseous exchange was occurring. The peroxide was tipped without removing the flasks from the bath to avoid upsetting thermal equilibration. Catalase activities were compared on the basis of rates of O_2 evolution during the first few minutes of the reaction. Catalytic activity against hydrogen peroxide of catalase, hematin, and charcoal was determined by substituting these chemicals for the cells in the concentrations described below.

Attempts to demonstrate H_2O_2 production in *R. quintana* were based on the catalase-aminotriazole method of Cohen and Somerson (3). The primary incubation mixtures (2 ml) contained either *R. quintana* or *Diplococcus pneumoniae* cells at equal densities of 0.40 (absorbance at 725 mm, 1 cm), either 8 mM sodium succinate or 6 mM glucose as the respective energy sources, 2,500 units of catalase per ml, 50 mM 3-amino-1,2,4-triazole, 10 mM sodium phosphate buffer, and 140 mM NaCl, *p*H 7.0. Incubation was at 37 C. Samples (0.1 ml) were removed at 0, 30, and 60 min for analysis of residual catalase activity as described by Cohen and Somerson (3).

RESULTS

Growth response of R. quintana to various levels of filtered hematin. A graded growth response was obtained when increasing concentrations, between 0 and 20 μg/ml, of hematin were added to a basal liquid medium containing brain-heart infusion, Thiotone, starch, and succinate. Some slight growth was obtained in the absence of added hematin on the first passage although growth, as measured both by rate of increase and by total amount of organisms, increased with increasing amounts of hematin. By the third passage, the dependency of growth on hematin concentration became more pronounced. Thus, no growth was obtained in the absence of hematin, and the most pronounced differential response was seen in the range of 0 to 8 μg/ml (Fig. 1). Both growth rate and the maximum population attained are significantly affected by hematin levels. Hematin appears to be essential for the growth of *R. quintana* in this synthetic medium.

Growth response of R. quintana to various levels of catalase, pyruvate, charcoal, and reducing compounds. *R. quintana* was transferred three times in crystalline catalase at levels of 0, 200, 400, 800, 1,200, and 1,600 units/ml. By the third transfer, no growth was observed at the lower levels, but at 1,200 and 1,600 units/ml some slight growth was observed. Hematin levels of 2 and 12 μg/ml were run concurrently as controls (Fig. 2). Similar growth experiments were conducted with potassium pyruvate (3 and 30 mM), animal charcoal (0.05, 0.10, and 0.20 mg/ml), sodium sulfite (0.8 and 1.6 mM), and sodium thioglycolate (0.8 and 1.6 mM) as substitutions for hematin. No growth was obtained with any of these materials.

Ability of hematin, sodium pyruvate, and charcoal to degrade hydrogen peroxide. Hematin, sodium pyruvate, and charcoal were all active against H_2O_2 to some degree. Table 1 shows that on a molar basis hematin is much more active than the other two substances.

Catalase activity in R. quintana. When washed cell suspensions of *R. quintana* and *E. coli* were compared, at equal absorbance values, in their ability to degrade H_2O_2, it was observed that only 4 μliters of O_2 were released by *R. quintana* as compared to 143 μliters by *E. coli* (Table 2). Because the amount of O_2 released in the presence of *R. quintana* was no more than in the corresponding control, it was concluded that *R. quintana* displayed little or no catalase-type activity.

Hydrogen peroxide secretion by R. quintana and D. pneumoniae. *R. quintana* and *D. pneumoniae* were analyzed for H_2O_2 secretion by using the catalase-aminotriazole technique. When employed at equal cell densities, *R. quintana* showed no evidence for H_2O_2 production, whereas *D. pneumoniae* was quite active, even when diluted 10-fold (Table 3).

Growth cycle of R. quintana in a liquid medium. Studies were conducted to determine

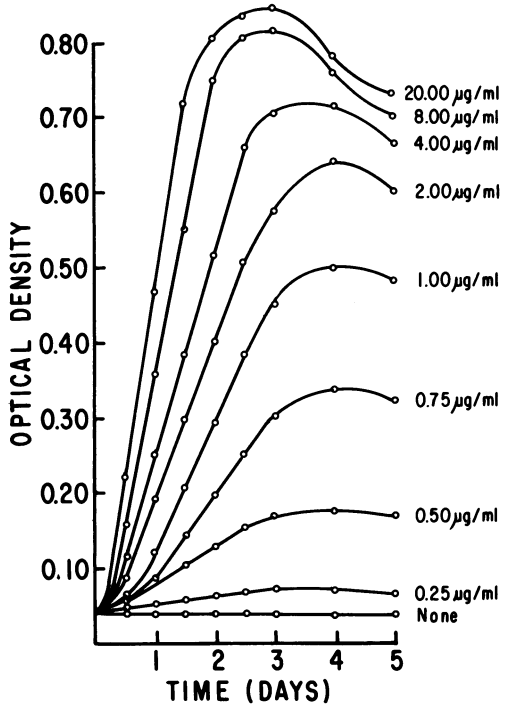

FIG. 1. *Growth response of Rickettsia quintana on third transfer to various levels of hematin.*

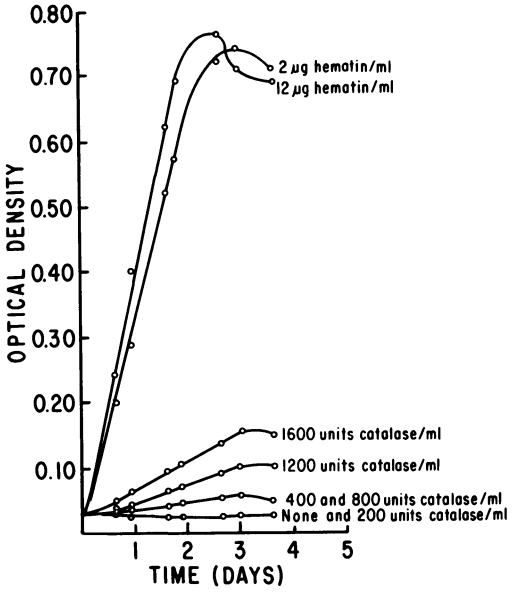

FIG. 2. *Growth response of Rickettsia quintana on third transfer to various levels of hematin and catalase.*

(i) the minimal inoculum size required to initiate growth, (ii) the generation time, (iii) the maximum viable population attained, (iv) the length of the stationary phase, and (v) the slope of the logarithmic death phase in the liquid growth medium.

Unwashed logarithmic phase cells which had been grown in the standard hematin medium were serially diluted from 10^{-1} through 10^{-8} in the same medium. Duplicate viable cell counts were made of each dilution before further incubation. Growth occurred after incubation in a roller drum from 10^{-1} through the 10^{-4} dilution only, with growth at 10^{-4} marginal; no growth occurred over the dilution range of 10^{-5} to 10^{-8} (Fig. 3). Plate counts indicated that the minimal inoculum size to initiate growth was thus around 10^5 cells/ml (final concentration). Growth was followed for each dilution tube both by periodic turbidity readings (Fig. 3) and by plate counts (Fig. 4). Correlation with plate counts indicated that a population of approximately 10^8 cells/ml is required for minimum measurable turbidity. A generation time of 10 hr was calculated from the data shown in Fig. 4. Other aspects of the growth cycle can be determined from Fig. 4.

Effect of temperature on growth or survival of R. quintana. The temperature range for growth was examined. Although the lag phase was somewhat shorter at 35 C than at 33 C, a higher cell population was attained at the latter temperature. A slower growth rate and a substantially lower yield were obtained at 37 C as compared with either 33 C or 35 C. Essentially no growth was obtained below 28 C or above 39 C, with only minimal growth at the latter temperature (Fig. 5). After incubation at 22 C for 5 days, no turbidity changes were ob-

TABLE 1. *Degradation of H_2O_2 by hematin, sodium pyruvate, and charcoal*

Substance	Quantity	Degradation of H_2O_2 (µmoles/hr)
Hematin	0.32 µmole	20[a]
	0.16 µmole	11
	0.08 µmole	6
Potassium pyruvate	10.0 µmole	7[b]
	5.0 µmole	4[b]
	2.5 µmole	2[b]
Charcoal	5.0 mg	8
	1.0 mg	2

[a] All of the peroxide added (20 µmoles) was degraded at 1 hr.

[b] Reaction ended spontaneously in less than 1 hr at the levels shown.

TABLE 2. *Catalase activity in Rickettsia quintana and E. coli*

Time (min)	Microliters of oxygen produced (cumulative)		
	Control (no cells)	*R. quintana*[a]	*E. coli*[a]
1	0[b]	1	16
2	1	1	51
3	2	3	86
4	2	4	115
5	4	4	143

[a] Both cell suspensions were adjusted to the same optical density, $OD_{725\ nm}$ (1 cm light path) = 3.0.
[b] Microliters of O_2 produced in the presence of 20 μmoles of H_2O_2.

TABLE 3. *H_2O_2 generation by Rickettsia quintana and Diplococcus pneumoniae as measured by the catalase-aminotriazole technique*

Time (min)	Per cent inhibition of catalase activity by			
	Control (no cells)	*R. quintana* (1× concn)	*D. pneumoniae*	
			(1× concn)	(0.1× concn[a])
30	0	3	99	76
60	0	3	99	98

[a] *D. pneumoniae* was used at a 1:10 dilution of the cell concentration used for *R. quintana* and for the previous *D. pneumoniae* determination.

served, although some growth did occur after a lag period on transfer to 33 C.

Effect of succinate and glutamate on growth. When either 17 mM sodium succinate or 14 mM sodium L-glutamate was added to the hematin basal medium (without succinate), a much higher growth level was attained than in their absence (Fig. 6).

DISCUSSION

Among microorganisms requiring hematin for growth, two main functions for hematin have been identified: (i) as a precursor for heme-protein synthesis or (ii) as a catalyst for degradation of H_2O_2. Lascelles (12) indicated that *H. influenzae* required hematin for the first reason whereas organisms such as *P. pestis*, *B. pertussis*, or the catalase-negative strains of the tubercle bacillus required it for the latter. The latter organisms require levels of hematin (1 to 5 μg/ml) which are 10 to 100 times those of *H. influenzae*. A catalytic role for hematin is particularly well documented in the case of the isoniazid-resistant, catalase-negative strains of the tubercle bacillus (4, 7, 11).

From the data presented in this report, it appears that *R. qunitana* requires hematin as a

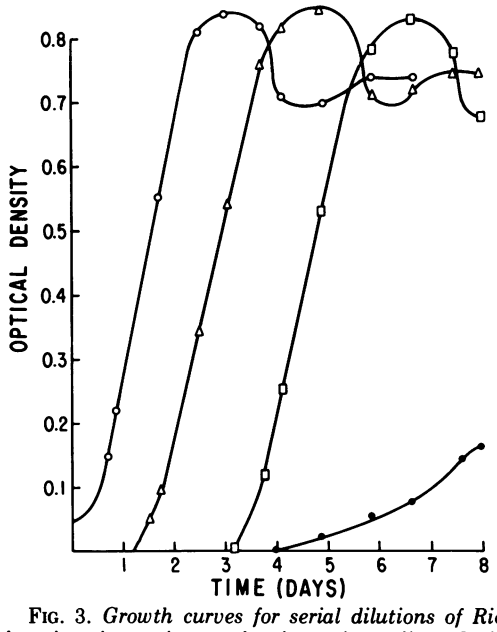

FIG. 3. *Growth curves for serial dilutions of Rickettsia quintana in complete hematin medium. Serial 10-fold dilutions were prepared from a logarithmic phase culture with an absorbance of 0.26. Symbols: (○) 10^{-1} dilution; (△) 10^{-2} dilution; (□) 10^{-3} dilution; (●) 10^{-4} dilution.*

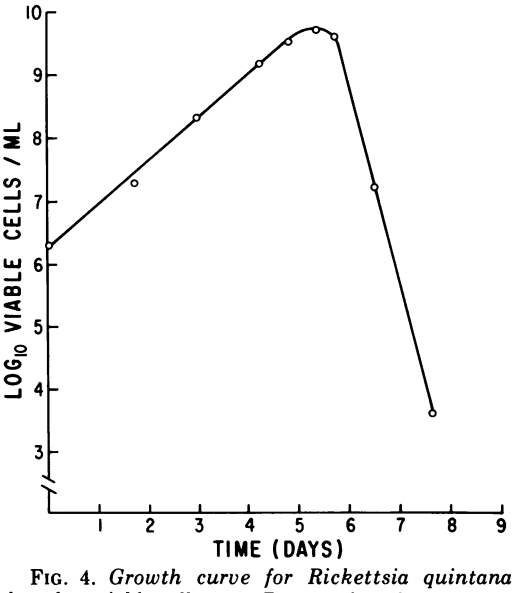

FIG. 4. *Growth curve for Rickettsia quintana based on viable cell count. Data are based on the 10^{-3} dilution shown in Fig. 3.*

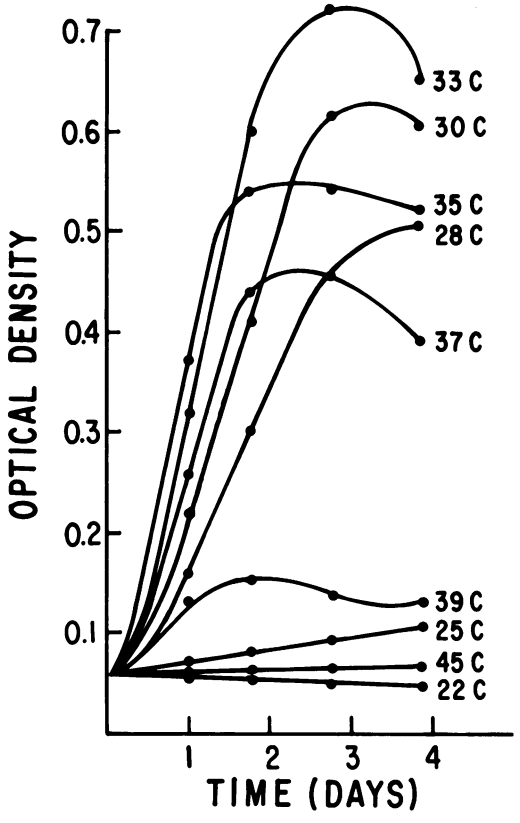

FIG. 5. *Effect of temperature on growth of Rickettsia quintana.*

precursor in the synthesis of various hemeproteins. This conclusion is based on the following observations.

(i) The maximum level of growth attained, as well as the growth rate, was dependent upon hematin concentration, suggesting that hematin was a limiting growth factor in this medium.

(ii) In the organisms previously mentioned as requiring hematin for their catalytic function, a number of compounds have been shown to substitute for hematin in this regard. These include catalase, sodium pyruvate, charcoal (4, 5, 9), and certain reducing substances such as sodium sulfite or sodium thioglycolate (3, 9). The amount of catalase activity (1,200 units/ml) required to show even a minimal growth response by *R. quintana* was approximately 100-fold higher than that giving a maximal growth response in the catalase-negative strains of the tubercle bacillus (4). In addition, if the mass and catalytic activity of hematin are equated, it is seen that 10 μg is equivalent to only 0.05 units of activity. It seems possible that the catalase was contaminated with a small amount of free hematin, which at higher catalase concentrations was sufficient to permit some growth. None of the other compounds was able to substitute for hematin in the growth of *R. quintana* when employed at levels shown to be satisfactory in the growth of the previously mentioned organisms. It is possible that the redox potential was unfavorable for the highly aerobic *R. quintana* in the presence of sodium sulfite or thioglycolate, although the quantities employed were considerably smaller than those present in the usual media designed to produce anaerobic conditions. The inability of the other hematin substitutes to support growth is best explained by the absolute need for hematin as a building block in heme-proteins.

(iii) *R. qunitana* does not appear to produce H_2O_2, thus eliminating the requirement for a

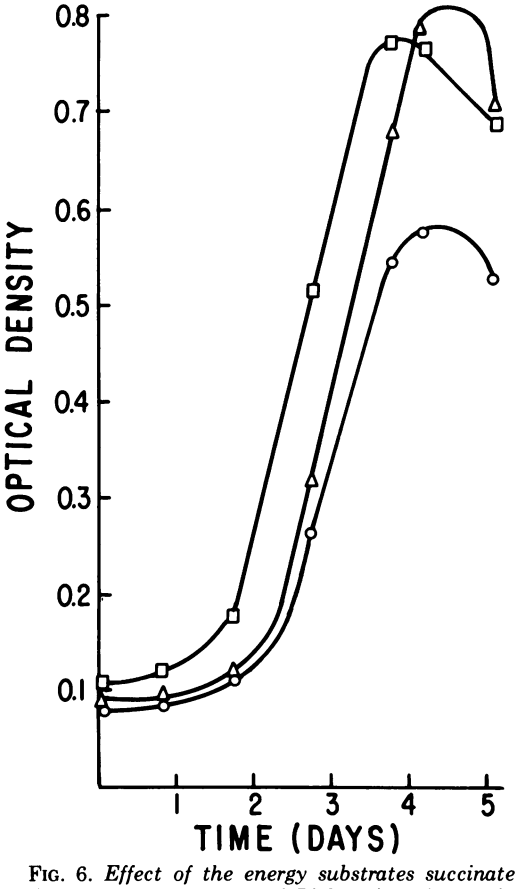

FIG. 6. *Effect of the energy substrates succinate and glutamate on growth of Rickettsia quintana in basal liquid medium. Symbols:* (○) *basal medium plus hematin;* (△) *basal medium plus hematin and 17 mM succinate;* (□) *basal medium plus hematin and 14 mM L-glutamate.*

mechanism to degrade H_2O_2. The catalase-aminotriazole method is a new procedure for measuring the secretion of H_2O_2, based upon an H_2O_2-dependent inhibition of catalase by 3-amino-1,2,4-triazole (3). The conversion of an H_2O_2 secretion rate into a catalase inhibition rate amplified a relatively small molar concentration of H_2O_2. Information about H_2O_2 production by other microorganisms is essentially limited to that obtained by older techniques and includes the standard horseradish peroxidase method with *o*-dianisidine as substrate (3) or the polarographic method (15). This new sensitive method provides the means by which H_2O_2 production can be studied, even in organisms with peroxidase activity, such as the mycoplasma (3). Further studies on H_2O_2 production with the obligately intracellular rickettsiae and the facultatively intracellular bacteria such as brucellae or *Listera* should help clarify the relationship of this phenomenon to bacterial or rickettsial intracellular parasitism.

(iv) *R. quintana* was demonstrated to be catalase-negative, a somewhat unusual finding since most bacterial species produce catalase. Nevertheless, the absence of catalase is consistent with the failure of this organism to produce H_2O_2. Molland (15), in his monograph on bacterial catalase, summarized the previous studies of others in addition to some findings of his own. Catalase-negative organisms are mostly limited to the genus *Clostridium* and to the *Lactobacteriaceae*, including the genera *Lactobacillus*, *Streptococcus*, and *Pneumococcus*, which also have an essentially anaerobic metabolism even in the presence of atmospheric oxygen. That obligately aerobic organisms may be catalase-negative is exemplified by the catalase-negative strains of *M. tuberculosis*.

A number of investigators are currently involved in the study of the biochemistry of the phagocytic process, particularly as it occurs in the leukocyte (17). There is mounting evidence that H_2O_2 is involved in this process operating in conjunction with host-produced peroxidases. Klebanoff (17) has reported that in chronic granulomatous disease a metabolic defect exists in which the lysosome fails to rupture. Thus, certain catalase-producing gram-negative bacteria are resistant to intracellular killing and frequently present serious clinical problems. However, catalase-negative, H_2O_2-producing organisms, such as the streptococcus or pneumococcus, can be killed in these defective leukocytes, presumably through the mediation of H_2O_2 produced by these bacteria. Intracellular killing by macrophages presumably involves somewhat different mechanisms. If the obligately intracellular rickettsiae show the same pattern as *R. quintana* and turn out to be catalase-negative and H_2O_2-negative, it would be tempting to speculate on the relationship of this condition to their ability to survive at least for a time within a phagocytic vacuole (Andrese and Wisseman, *personal communication*).

The large (10^5 cells/ml) inoculum required to initiate growth of *R. quintana* in our liquid medium (Fig. 3) cannot be explained on the basis of carry-over of nutrients from a qualitatively different medium because *R. quintana* has been serially passed innumerable times in this same medium. Halman et al. (8) have described a similar high threshold inoculum requirement for *Francisella tularensis*. The long generation time of 10 hr described here for *R. quintana* is certainly most unusual in relation to other pathogenic, extracellular or facultative intracellular bacteria, particularly for a rich, complex medium like the brain-heart infusion employed in these studies, and is probably exceeded only by the pathogenic mycobacteria. It is, however, closely similar to the generation time of *R. tsutsugamushi* in a cell culture system where a threefold increase in 24 hr was described (2). Mason (13), however, reported a generation time of 4.5 hr for *R. quintana* when grown in a medium containing fetal calf serum, yeast extract, and TES buffer. A possible explanation for this difference might be that some component was lacking in our medium which *R. quintana* was capable of synthesizing and that this synthetic pathway was rate-limiting.

The range of temperature for the growth of *R. quintana* extends from approximately 28 to 37 C, with an optimum near 33 C. A parallel phenomenon has been observed in the growth of the obligately intracellular rickettsiae in cell culture (2) and embryonated eggs (18), where a temperature several degrees below 37 C seemed preferential. Although in the latter case the temperature effect on the host cell may enter into the interpretation of this phenomenon, it is consistent with proliferation at the lower temperatures which often exist in arthropod vectors.

The growth stimulus provided by L-glutamate and succinate might be explained by the fact that *R. quintana* is unable to oxidize glucose but can oxidize glutamate and succinate efficiently (10). Moreover, if *R. quintana* is unable to synthesize glutamate and thus requires an exogenous source for protein synthesis, the addition of glutamate, above the

level provided by the brain-heart infusion, or the addition of succinate could have a sparing effect on glutamate available for protein synthesis.

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