STUDY ON GROWTH OF RICKETTSIAE

V. Penetration of Rickettsia tsutsugamushi into Mammalian Cells in Vitro

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The ability of an intracellular parasite to invade a cell is one of the most critical factors in the events leading to overt infection. Studies on the mechanisms involved in this process have been confined chiefly to the bacteriophages (1) and the hemagglutinating animal viruses (2). Little detailed information is available on the interaction of intracellular animal pathogens and the host cells which support their subsequent multiplication. The studies to be presented describe one such *in vitro* relationship between the Karp strain of *Rickettsia tsutsugamushi*, the agent of scrub typhus, and the MB III strain of mouse lymphoblasts.

The rickettsiae are generally considered to be obligate intracellular parasites, differing from the smaller animal viruses in that they possess a high degree of structural complexity (3), an independent metabolism (4, 5), and the ability to multiply by transverse binary fission (6). In addition, there has been an increasing body of information which has correlated the metabolic activities of these organisms with such biological attributes as infectivity, the hemolysis of erythrocytes, and toxicity for small rodents (7, 8). These studies suggested a unique relationship between rickettsiae and their host cells and prompted an investigation of the early phases of infection; *i.e.*, the penetration of rickettsiae into susceptible mammalian cells. Evidence will be presented to suggest the active participation of rickettsiae in the invasion of the host cell.

Materials and Methods

Cell Strains.—The MB III strain of mouse lymphoblasts was used in the major portion of these studies. They grow as free-floating cells in the nutritive medium which facilitates enumeration and the preparation of replicate cultures. The derivation and methods of cultivation of the cell line were reported in a previous publication (9). A few comparative experiments were performed employing the L strain of mouse fibroblasts (10). Both lines of cells are maintained in this laboratory in a growth medium composed of the following: Gey's

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balanced salt solution (BSS), 50 per cent; beef embryo extract (1:2 dilution of original), 10 per cent; horse serum, 40 per cent; phenol red, 0.0025 per cent; and penicillin and streptomycin, 100 units/ml. and 20 μg./ml., respectively. All constituents of the medium, except the antibiotics, were obtained from Microbiological Associates, Inc., Bethesda.

Rickettsial Strain.—The egg-adapted line of the Karp strain of *Rickettsia tsutsugamushi* was used exclusively in the present investigation (11). Two pools consisting of 20 per cent suspensions of infected yolk sacs were prepared as seed material and stored at −70°C. in 2 ml. and 10 ml. amounts. Pool I was prepared from yolk sacs of the 94th passage and Pool II from the 100th passage.

Some of the results to be reported are directly related to the survival of the rickettsiae in the various media under study. The stability or loss of infectivity of the organisms suspended in these cell-free fluid media was determined by titrations in mice. Serial tenfold dilutions of the rickettsial suspensions, prepared as for the penetration studies, were made in Snyder I diluent (12), and each of six 14 to 16 gm. Swiss Bagg mice were inoculated intraperitoneally with 0.2 ml. of the proper dilution. The LD_{50}, determined by the method of Reed and Muench, was calculated on the basis of deaths occurring over a period of 28 days.

Experimental Procedure for the Study of Penetration.—The basic procedures employed to study the penetration of *R. tsutsugamushi* into the MB III cell were the same in all experiments. In general, a partially purified rickettsial inoculum was added to a standardized pool of MB III cells and incubated in a roller drum. At various time intervals aliquots were removed, the cells concentrated by low speed centrifugation, and subsequently smeared and stained. The stained cells were examined microscopically to determine the percentage of cells invaded by rickettsiae and the severity of infection. Detailed procedures for the preparation of the MB III cells, the rickettsial inoculum, and the quantitation of penetration are presented below.

Preparation of the MB III Cells.—Several days prior to an experiment the number of MB III cultures was increased by subculturing the existing stock tubes. On the day of the experiment, the cultures were pooled and the number of cells per milliliter determined by hemocytometer counts. Aliquots of the pool containing 6×10^6 cells were pipetted into 15 ml. centrifuge tubes. The cells were sedimented by centrifugation at 1,000 R.P.M. for 5 minutes and the supernatant growth medium carefully removed. The packed cells were resuspended gently in 1.75 ml. of medium and transferred to 16 × 150 mm. test tubes. A rickettsial inoculum of 0.25 ml. suspended in the same medium used for the MB III cells was then added.

Preparation of Rickettsiae.—Depending upon the requirements of the experiment, appropriate amounts of the frozen 20 per cent yolk sac homogenate were thawed rapidly in a 37°C. water bath. The suspension was diluted to 10 per cent with an equal volume of diluent and clarified by centrifugation at 1,000 R.P.M. for 5 minutes. The composition of the diluent employed was contingent on the design of the experiment. Following the low speed centrifugation the midzone which contained rickettsiae and small yolk sac particles was carefully removed and transferred to sterile 8 ml. lusteroid tubes. This material was centrifuged at 13,000 R.P.M. (Servall, model SS-1) at 4°C. for 15 minutes. The supernatant fluid was decanted and the walls of the tube thoroughly swabbed with sterile cotton applicators. The tightly packed rickettsial sediment was homogenized with a glass rod and resuspended in the same diluent to contain approximately 1.68×10^8 organisms per ml. This constituted the rickettsial inoculum which was added immediately to the cell suspension.

At *zero* time a 0.25 ml. aliquot of the rickettsial inoculum was added to roller tubes containing 6×10^6 MB III cells suspended in 1.75 ml. of medium. The final volume of the experimental tubes was always 2.0 ml. The tubes were tightly sealed with rubber stoppersd an placed in a roller drum (Wyble Engineering Development Corp., Silver Spring, Maryland) at 12 R.P.M. Unless otherwise stated, the cultures were incubated at 37°C.

After 30 and 120 minutes of incubation, 0.2 ml. of the suspension was pipetted into 13 x 100 mm. tubes and the cells sedimented at 800 R.P.M. for 3 minutes. The supernatant fluid containing yolk sac particles and extracellular rickettsiae was removed, and the pellet of cells was resuspended in the residual fluid. The drop of cell suspension was placed on a glass slide, smeared as for blood films, and allowed to air dry. The smears were subsequently fixed with absolute methanol and stained with a dilute Giemsa stain (pH 6.8). The rickettsiae, when examined with an oil immersion objective, stained a deep purple as opposed to the pale blue cytoplasm of the MB III cell. Because of the characteristic localization of *R. tsutsugamushi* in the juxtanuclear region little difficulty was encountered in determining the intracellular or extracellular position of individual rickettsiae. Only those organisms which were well within the cytoplasmic border of the cell were considered for purposes of computation.

Determination of Penetration Index.—Each of two observers examined 100 well stained cells on separate smears to determine the percentage of cells invaded by rickettsiae and the severity of the infection. The average of these data was employed to evaluate the degree of infection by means of the following key:

		Multiple for determining penetration index
H (heavy)	>20 rickettsiae/cell	3
M (moderate)	6–20 rickettsiae/cell	2
L (light)	1–5 rickettsiae/cell	1
O	No visible rickettsiae	0

In order to quantitate the number of rickettsiae per host cell, each category, *i.e.*, *H*, *M*, and *L*, was scored as above to obtain a Penetration Index (P.I.).

$$\text{Penetration Index} = 3\ (\text{per cent H}) + 2\ (\text{per cent M}) + 1\ (\text{per cent L})$$

The penetration index (hereafter referred to as P.I.) is an estimate of the number of rickettsiae observed in the host cell per unit time. The validity of the multiples was determined by counting the actual number of rickettsiae per cell in each of the categories. With the number of rickettsiae employed in all of the following experiments, the "M" and "L" multiples were accurate; *i.e.*, there were approximately twice the number of organisms in the *moderate* as in the *light* category. The "H" or *heavy* multiple was inaccurate, but since this degree of infection was so rarely encountered, it did not significantly influence the P.I.

Preliminary experiments revealed that the P.I. depended upon the concentration of both the rickettsiae and MB III cells. When different multiplicities of infectious particles (0.6 to 7) to MB III cells were studied, while maintaining a constant MB III cell population of 3×10^6 per ml., the P.I. varied as an approximately linear function of the number of rickettsiae. It was necessary, therefore, to maintain a standard multiplicity of rickettsiae to host cells in each experiment. The two infected yolk sac pools contained slightly different numbers of organisms as determined by mouse titrations and, thus, yielded different P.I.'s. The inoculum prepared from pool I contained $10^{7.6}$ mouse LD_{50}'s and from pool II, $10^{7.5}$ mouse LD_{50}'s. This represented a multiplicity of 7 rickettsiae/MB III cell and 5 rickettsiae/cell, respectively. Although pool II yielded slightly lower P.I.'s, the quantitative effects of the various factors to be reported were similar when either pool I or II was employed. The results of the following experiments are expressed in terms of P.I. or as per cent of the P.I. obtained in the control culture.

In several instances in which the multiplication of rickettsiae was examined, the cultures were maintained for an additional 48 to 72 hours. After the 2 hour penetration period, the

cell suspension was diluted with complete medium so that the final concentration of cells was 1×10^6 per ml. Since infected cells continue to multiply, colchicine (0.1 μg./ml.) was added to the medium to stabilize the host cell population (9). Aliquots of the suspension were removed at 24 hour intervals and the cells smeared and stained. Rickettsial multiplication was determined by microscopic observations.

RESULTS

The Penetration of R. tsutsugamushi (Karp Strain) into the MB III Cell in Complete Medium.—The initial studies were conducted in the complete medium (see Materials and Methods) which represented an optimal environment for the survival of both the rickettsiae and MB III cells. A fresh inoculum of rickettsiae which was purified in complete medium was added to the cells suspended in the same menstruum. The top line of Fig. 1 shows a plot of the mean P.I.'s obtained from eight separate preparations of cells and rickettsiae (pool I). The standard deviation of each sample (30, 60, and 120 minutes) was within ± 5 P.I. units. As evidenced by the slope of the line, the rate of penetration was rapid during the early phase of the experiment and gradually became asymptotic. The value at 30 minutes was approximately 80 per cent of that obtained after 120 minutes of incubation. In other experiments not presented, in which samples also were removed after 5, 10, and 15 minutes of incubation, it was clear that the rate of penetration remained strictly linear only for the first 15 minutes. After 120 minutes no significant increase in the P.I. occurred and the values remained constant until the organisms began to multiply.

The upper broken line shows the percentage of cells which contained visible rickettsiae. Although only a few per cent more cells became infected after the first 30 minutes, the steady increase in the P.I. up to 120 minutes indicated that an increasing number of rickettsiae continued to invade cells which were already infected. Experiments in which a second inoculum was added to the system after 120 minutes of incubation showed that the organisms invaded the previously infected cells.

The Influence of Rickettsial and Host Cell Viability on the Penetration Process.—The "activity" or "viability" of the rickettsiae employed in these studies is expressed in terms of mouse infectivity. Procedures which "inactivated" the rickettsial inoculum resulted in a marked reduction in the P.I. Examination of smears prepared from cell suspensions to which non-viable rickettsiae were added failed to show any intracellular organisms (Fig. 1). There were, however, large numbers of extracellular rickettsiae present, which exhibited the same morphological and tinctorial properties as seen in untreated, viable suspensions.

The following methods were employed to inactivate the rickettsial inoculum: (*a*) heat—incubation in a 56°C. water bath for 30 minutes; (*b*) ultraviolet irradiation—a 1.0 ml. aliquot was placed in a 60 mm. Petri dish and irradiated for 5 minutes with an 8 watt germicidal lamp (2537 A) at a distance of 13 cm.; and (*c*) formalin—incubation for 15 minutes at 23°C. in the presence of 0.1 per cent formaldehyde U.S.P. Prior to the inoculation of the cells, the

formalin was removed by centrifugating the rickettsiae and resuspending the pellet in complete medium. A control, from which formalin was omitted, was treated in the same manner.

Mice injected with each of these undiluted suspensions did not develop scrub typhus. In addition, the cultures inoculated with the same suspensions were maintained at 37°C. for 72 hours, and smears prepared at 24 hour intervals over this period did not reveal any intracellular rickettsiae.

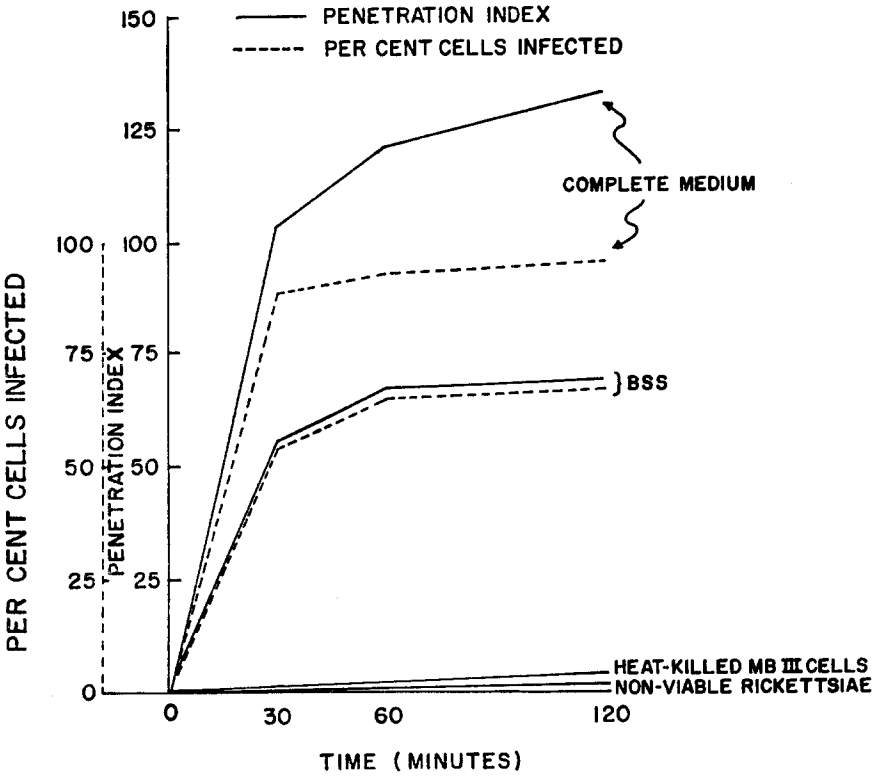

FIG. 1. The penetration of *R. tsutsugamushi* in complete medium and in balanced salt solution respectively, and the influence of host cell and rickettsial viability on the process.

Identical results were obtained when the strain L cell was employed under the same conditions.

Similar studies were conducted to determine the effects of heat and ultraviolet irradiation on the MB III cell. Cell suspensions which were incubated at 56°C. for 5 minutes failed to become infected upon the addition of a standard rickettsial inoculum (Fig. 1). In contrast, irradiation of the cells for 90 seconds, under conditions similar to that described above, did not alter their susceptibility to infection. It was of interest that although the amount of irradiation

received by these cells was sufficient to prevent subsequent multiplication, they were still capable of supporting the growth of rickettsiae as evidenced by the increased numbers of intracellular organisms after 48 hours of incubation.

It appears, therefore, that a major requirement for the penetration process is related to the "viability" of the invading rickettsiae. Although the integrity of the host cell is of obvious importance, certain fundamental properties, *e.g.* the ability to multiply, are not necessary for the invasion or subsequent multiplication of the rickettsiae.

The Role of the Ionic Composition of the Medium on the Penetration of R. tsutsugamushi into the MB III Cell.—The complete medium employed in the previously described studies consisted of a variety of complex macromolecules as well as small organic and inorganic compounds. In order to delineate some of the chemical requirements necessary for penetration, the medium was subdivided into its various components and penetration was allowed to proceed in the presence or absence of these constituents. Invasion of the cells suspended in balanced salt solution (BSS) alone was first examined.

The BSS employed in all experiments had the following composition:—

Compound	Concentration
	M
NaCl	0.137
KCl	0.005
$CaCl_2$	0.001
$MgCl_2$	0.00103
Na_2HPO_4	0.00084
KH_2PO_4	0.00018
Glucose	0.011
$NaHCO_3$	0.0029

Phenol red was added in a final concentration of 0.0025 per cent and the pH was adjusted to 7.2. Both the rickettsial inoculum and cell suspension were prepared in this medium prior to mixing and incubation at 37°C.

As shown in Fig. 1 when the cells and rickettsiae were suspended in BSS alone, the P.I. was about 50 per cent of that of the complete medium, which also contained horse serum and beef embryo extract. The variation between the per cent of cells infected and the P.I. was minimal, indicating that the majority of cells were lightly infected. After 60 minutes of incubation, few, if any, additional rickettsiae were found within the cells. The addition of a rickettsial inoculum prepared in BSS to MB III cells suspended in complete medium, gave results identical with the control prepared entirely in the complete medium. This indicated that the rickettsiae were not significantly damaged when purified in BSS.

Further studies were conducted to determine the constituents of BSS necessary for rickettsial invasion. Fig. 2 A pertains to this study. As shown by the

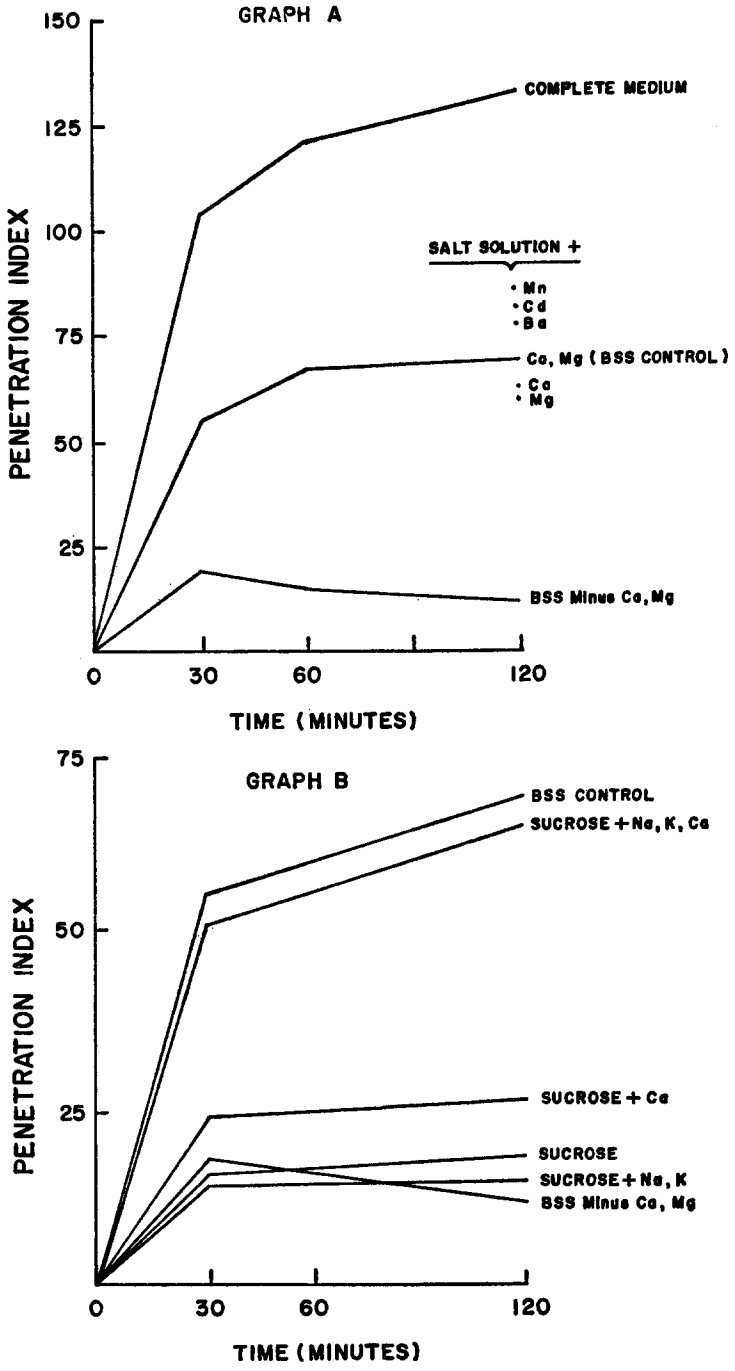

FIG. 2. The role of the ionic composition of the medium on the penetration of *R. isutsuga-mushi* into the MB III cell.

lower line penetration was significantly depressed when the divalent cations, calcium and magnesium, were omitted from the BSS. The composition of the salt solution was exactly the same as described above except for the omission of calcium chloride and magnesium chloride. This solution was used as the diluent for the purification of the rickettsial inoculum and the preparation of the MB III cell suspension.

The effect of the divalent cations, however, was not specific, as evidenced by the ability of manganese, barium, or cadmium at the same final concentration (0.001 M) to replace this requirement. Similarly, calcium and magnesium when singly present, served equally well.

The addition of disodium versenate (Bersworth Chemicals, Framingham, Massachusetts) at a final concentration of 0.001 M to the divalent cation-free media had no further effect on the P.I., and suggests the absence of significant amounts of available divalent cations carried over from the rickettsial inoculum and the MB III cells.

Fig. 2 B presents the results of experiments in which sucrose was employed to maintain the isotonicity of a non-ionic environment. In the sucrose solution alone, the P.I. was the same as that obtained with the salt solution devoid of the divalent cations. The addition of both sodium and potassium chloride at a final concentration of 0.13 and 0.005 M, respectively, to the sucrose solution, produced no increase in penetration. When calcium chloride (0.001 M) was added, there was a slight increase which may or may not be significant. In contrast, the presence of all three cations, raised the P.I. to that of the BSS control. The absence of phosphate ions in the sucrose solution indicated that no exogenous supply of this substance was required.

It appears, therefore, that both the monovalent and divalent cations are required for the penetration of *R. tsutsugamushi* (Karp strain) into the MB III cell. In the case of the divalent cations this represents more than a contribution to the ionic strength of the medium and can be affected by a variety of cations, two of which (barium and cadmium) have no known interrelations with the metabolism of either the parasite or the host cell.

The Ability of Albumin to Replace Horse Serum and Beef Embryo Extract.—The higher penetration values obtained with the complete medium as compared with BSS indicated that a factor or factors present in the horse serum and beef embryo extract were required for maximal invasion. These factors were studied by adding various compounds to BSS and comparing the resulting P.I.'s with that of the complete medium control.

Table I shows that the addition of 1 per cent bovine serum albumin (fraction V. Armour) to BSS yielded indexes which were equal to or slightly higher than the complete medium control. Penetration was depressed, however, when the concentration of albumin was increased to 3 per cent (a concentration of protein which is similar to that of the complete medium). Although the mech-

anism of the augmenting effect of albumin is not clear, previous studies (13) have shown albumin to have a stabilizing effect on the survival of many species of rickettsiae including the Karp strain of *R. tsutsugamushi*. Similar studies conducted in the present investigation also demonstrated the protective effect of 1 per cent albumin on the survival of *R. tsutsugamushi* in cell-free media.

The Effect of Specific Immune Serum.—Both opsinization and neutralization of rickettsiae by specific immune serum have been described by a number of investigators (14–16). Analogous studies were performed in order to examine

TABLE I

Effect of Albumin on the Penetration of R. tsutsugamushi (Karp Strain) into MB III Cells*

Medium	Penetration index after incubation at 37°C.		
	30 min.	60 min.	120 min.
BSS	55	67	70
1 per cent albumin‡	114	135	138
3 " " "	80	88	98
Complete	103	121	133

* Pool I.

‡ The albumin was dissolved in BSS and adjusted to pH 7.2 with N KOH.

the influence of immune serum on the penetration process. The experimental procedure is outlined below.

Suspensions of pool I were purified in complete medium by the previously described methods. An aliquot of 0.4 ml. was incubated with 0.1 ml. of undiluted serum for 30 minutes in a 37°C. water bath. From this, 0.25 ml. was removed and added to the standard number of MB III cells. Human, monkey, and rabbit convalescent scrub typhus sera were employed in the test, as well as the respective normal serum controls. Another tube contained the complete medium instead of serum as an additional control. The immune sera used contained high titers of complement-fixing antibody (1:160 to 1:320) when tested with a Karp strain soluble antigen.

All the tubes which had been preincubated at 37°C. showed essentially the same P.I., with a mean value of 59 (±2.5) as compared to 125 for the untreated control. No influence of specific antiserum was noted, either in increasing or decreasing the penetration of the organisms. It was clear, however, that the preincubation of rickettsiae for 30 minutes at 37°C. had a decided effect in lowering the P.I., and is probably related to the thermal inactivation of the organisms at this temperature.

The Effect of Temperature on Penetration.—The results of studies to determine the effect of temperature on the penetration process are presented in Table II. After equilibrating the rickettsial inoculum and cell suspension to the indicated temperatures, they were mixed and placed in a roller drum at

the same temperature. Following the standard procedures, aliquots were removed and the cells smeared and stained.

The results show that the P.I. was influenced by the temperature of incubation. At lower temperatures fewer rickettsiae entered the host cells. However, the per cent reduction in P.I. varied with the medium employed so that a more striking effect was noted in the non-protein–containing medium (BSS) than in either complete medium or albumin. This effect is as yet unexplained.

A comparative experiment on penetration employing the strain L cell suspended in complete medium resulted in a 47 per cent reduction in the P.I. at 4°C.

TABLE II

Effect of Temperature on the Penetration of R. tsutsugamushi (Karp Strain) into MB III Cells in Complete Medium, Albumin, and Balanced Salt Solution (BSS)

Rickettsial suspension	Medium	Temperature	Penetration index after incubation, 120 min.	Per cent of penetration index at 37°C.
		°C.		
Pool I	Complete	37	127	100
" "	"	27	107	84
" "	"	4	80	63
Pool II	Complete	37	92	100
" "	"	4	68	74
Pool II	Albumin*	37	91	100
" "	"	4	66	73
Pool II	BSS	37	58	100
" "	"	4	20	35

* 1 per cent albumin in BSS.

No irreversible change in the susceptibility of the MB III cell occurred at low temperatures, since preincubation of the cell suspension at 4°C. for 2 hours did not alter their ability to become infected when returned to 37°C. The low temperature (4°C.) employed in these studies aided in maintaining the viability of the organisms. Indeed, the incubation of rickettsial suspensions at 4°C. for 120 minutes in complete medium, 1 per cent albumin, and BSS resulted in no demonstrable loss of mouse infectivity.

Whereas the penetration of rickettsiae in complete medium was relatively temperature independent, their subsequent multiplication in the host cell was greatly influenced by the temperature of incubation. Cultures incubated at 37°C. for 72 hours exhibited marked rickettsial multiplication; similar cultures maintained at 27°C. showed only slight multiplication. In contrast, there was no increase in the number of organisms at 4°C.

Metabolic Factors Related to Penetration.—A variety of independent metabolic functions have been ascribed to the rickettsiae (4, 5, 17, 18). Such studies have demonstrated, in particular, the oxidation of a number of compounds related to the Krebs' cycle. One such substrate, glutamic acid, which is most actively oxidized by *Rickettsia mooseri* and *Rickettsia prowazekii*, also has been shown to help maintain the infectivity, toxicity, and hemolytic activities of purified suspensions when incubated *in vitro* (8, 13). Since the penetration of *R. tsutsugamushi* into the MB III cell was related to the "viability" of the parasite, it was important to study the effect of various metabolites which are intimately associated with the metabolism of the rickettsial cell.

The rickettsial inoculum and cell suspensions were prepared in BSS. Immediately prior to the penetration experiment, the various compounds to be tested were dissolved in BSS to give a 10 × concentrated stock solution. The pH was adjusted to 7.2 with 2 N potassium hydroxide. A 0.2 ml. amount of the stock solution was added to 1.55 ml. of an MB III cell suspension containing 6×10^6 cells. A standard rickettsial inoculum (pool II) contained in 0.25 ml. of BSS was added to make a final volume of 2 ml. and penetration allowed to proceed at 37°C.

The results are expressed as the P.I. and as per cent of the P.I. obtained in the BSS control. The standard deviation of eight BSS controls was within ±5 P.I. units.

All of the metabolites, except D-glutamic acid, were highly purified compounds obtained from commercial sources and employed without further purification. The specimen of D-glutamic acid was purified by means of L-glutamic decarboxylase and crystallized as the hydrochloride (19).

The results obtained with a number of amino acids are presented in Table III. L-glutamic acid produced a marked increase in the P.I., whereas the other amino acids were either without effect, or as in the case of aspartic acid, there was a reduction in the P.I. The Krebs' cycle intermediates which are oxidized by rickettsiae to a slight but demonstrable extent did not significantly influence penetration. The addition of diphosphopyridine nucleotide (DPN), a cofactor important for rickettsial oxidation of glutamic acid increased penetration slightly. When DPN and glutamic acid were both added to the system, the resulting P.I. was identical with that obtained with glutamic acid alone. Adenosinetriphosphate (ATP), which is considered to be an important source of rickettsial energy (20), was without effect.

The results of further studies which were conducted with glutamic acid and related compounds are presented in Table IV. Although the minimal quantity of glutamate required to increase penetration was not determined, it is clear that as little as 1 μg./ml. (0.00006 M) was still effective. The unnatural D isomer which is not oxidized by rickettsiae (21) was inactive and, indeed, depressed the P.I. when employed in similar concentrations. L-glutamine proved as effective as glutamic acid in increasing penetration. It is of interest that the

oxidation of glutamine by *R. mooseri* has recently been demonstrated by Hahn *et al.* (22).

Bovarnick and Miller (17), in studies concerned with rickettsial transamination, reported vigorous oxygen consumption with mixtures containing α-ketoglutaric and aspartic acids. This was thought to be the result of glutamic acid formation followed by its subsequent oxidation. Similar mixtures employed

TABLE III

Effect of Selected Metabolites on the Penetration of R. tsutsugamushi (Karp Strain) into MB III Cells

Metabolites	Final concentration in BSS	Penetration index after incubation at 37°C.		Per cent of BSS control*	
		30 min.	120 min.	30 min.	120 min.
	M				
L-Aspartic acid	0.006	22	23	54	45
L- " "	0.003	13	22	35	46
L-Glycine	0.006	34	40	83	78
L-Lysine	0.006	41	42	100	82
L-Arginine	0.006	26	32	63	63
L-Alanine	0.006	50	70	85	82
L-Glutamic acid	0.006	99	134	212	234
Sodium pyruvate	0.006	47	62	80	97
Succinic acid	0.006	67	71	114	111
Malic acid	0.006	57	58	97	91
Oxalacetic acid	0.006	46	60	78	94
Citric acid	0.006	43	59	73	92
α-Ketoglutaric acid	0.003	48	50	110	104
DPN‡	0.0005	64	84	128	140
ATP§	0.0001	44	51	86	93

* Individual values calculated from the BSS control of each experiment.

‡ Diphosphopyridine nucleotide.

§ Adenosinetriphosphate.

in the penetration system also caused a marked increase in the P.I. (Table IV). However, α-ketoglutaric and aspartic acids were without effect when employed separately (Table III). Only a minimal effect was noted when either alanine or glycine were used as amino group donors.

The Influence of L-Glutamic Acid on the Stability of R. tsutsugamushi (Karp Strain) and on the Susceptibility of the MB III Cell.—Although glutamic acid is the substrate most actively oxidized by rickettsiae it may also serve as a metabolite for the MB III cell. The enhancing effect of glutamic acid under the conditions of the penetration experiments, therefore, could conceivably be related to an effect on the rickettsiae and/or host cell. With this question in

TABLE IV

Effect of L-*Glutamic Acid and Related Compounds on the Penetration of R. tsutsugamushi (Karp Strain) into MB III Cells*

Metabolites	Final concentration in BSS	Penetration index after incubation at 37°C.		Per cent of BSS control*	
		30 min.	120 min.	30 min.	120 min.
	M				
L-Glutamic acid	0.006	99	134	212	234
L- " "	0.0006	100	132	208	228
L- " "	0.00006	76	98	158	175
D-Glutamic acid	0.006	22	31	46	53
D- " "	0.0006	23	32	48	55
L-Glutamine	0.006	104	130	217	224
α-Ketoglutaric acid +	0.003				
L-aspartic acid	0.003	81	108	170	186
α-Ketoglutaric acid +	0.003				
L-alanine	0.003	71	93	148	160
α-Ketoglutaric acid +	0.003				
L-glycine	0.003	51	67	106	116

* Values calculated from the BSS control of each experiment.

TABLE V

Effect of L-*Glutamic and* L-*Aspartic Acids on the Survival of R. tsutsugamushi (Karp Strain) at 37°C.*

Incubation at 37°C.	Medium					
	BSS		BSS + L-glutamic acid (0.006 M)		BSS + L-aspartic acid (0.006 M)	
hrs.	*log* LD_{50}	Δ *log* LD_{50}	*log* LD_{50}	Δ *log* LD_{50}	*log* LD_{50}	Δ *log* LD_{50}
0	7.5	—	7.5	—	7.5	—
1	6.3	1.2	7.1	0.4	5.6	1.9
2	4.3	3.2	6.4	1.1	3.2	4.3
3	<1.0	7.5	2.8	4.7	<1.0	7.5
4	<1.0	7.5	2.5	5.0	<1.0	7.5

mind experiments were performed to study the effect of glutamate on both members of the system.

The results of an experiment to determine the effect of L-glutamic acid and L-aspartic acid on the viability of *R. tsutsugamushi* in cell-free solutions are presented in Table V.

Aliquots of a standard rickettsial inoculum prepared in BSS were added to tubes containing (*a*) BSS, (*b*) 0.006 M L-glutamic acid in BSS, and (*c*) 0.006 M L-aspartic acid in BSS. The diluted suspensions which were considered as 10^{-1} dilutions of the original were placed in a roller drum at 37°C. Samples were removed at the designated times and titrated in mice.

It is evident from the tabular data that a rapid loss in infectivity occurred when the organisms were suspended in BSS and the L-aspartic acid solution. The titer had dropped 1,000-fold and 10,000-fold, respectively, after 2 hours of incubation, and no viable organisms were detectable after 3 hours. In contrast, the rickettsiae suspended in glutamic acid retained their infectivity for a longer period of time, with only a 10-fold reduction in titer at the end of 2 hours.

TABLE VI

Influence of L-Glutamic Acid on the Penetration of Pretreated Suspensions of R. tsutsugamushi (Karp Strain)

Tube	Treatment of rickettsial inoculum prior to addition to cells	MB III cells suspended in:	Penetration index after incubation at 37°C.
		Medium	120 min.
a	None*	BSS	48
b	None	L-glutamic acid‡	138
c	120 min. at 37°C. in BSS	BSS	17
d	120 min. at 37°C. in L-glutamic acid	BSS	81
e	30 min. at 25°C. in formalin§	L-glutamic acid	4
f	30 min. at 56°C. in BSS	L-glutamic acid	2

* Rickettsiae prepared immediately prior to addition to MB III cells.

‡ L-glutamic acid, when present, was at a final concentration of 0.006 M in BSS.

§ Formaldehyde U.S.P. was added to BSS to give a final concentration of 0.1 per cent. The formaldehyde was removed by sedimenting the rickettsiae at 13,000 R.P.M. for 15 minutes and resuspending in BSS.

Additional tests were performed to evaluate the effect of glutamic acid both on the maintenance of rickettsial viability and on penetration. Suspensions of rickettsiae which had been previously incubated at 37°C. for 120 minutes in the presence and absence of glutamic acid were added to freshly prepared MB III cells. The results presented in Table VI show that the P.I. was considerably higher when glutamate was present during the preincubation period (tube *d*). Lower values were obtained when the organisms were subjected to 37°C. temperature in BSS alone (tube *c*), and which had presumably suffered a greater loss in infectivity (see Table V). A comparison of tubes *c* and *d* with their respective untreated controls, *a* and *b*, revealed that preincubation even in the presence of glutamate resulted in a lower P.I. Table VI also illustrates that glutamic acid cannot effect the penetration of rickettsiae which have been rendered non-viable with either heat or formalin (tubes *e* and *f*).

It appears, therefore, that one function of glutamic acid in increasing penetration is related to its ability to maintain the viability of the organisms during the period of the penetration experiments.

Table VII summarizes the results of analogous experiments in which the effect of glutamic acid on the susceptibility of the MB III cell was studied. In this case the host cells were preincubated in BSS alone and BSS containing glutamic acid, prior to the addition of a freshly prepared rickettsial inoculum. It is apparent that incubation of the cells for 120 minutes at 37°C. in BSS (tube *c*) did not result in any significant alteration of the cells for subsequent rickettsial infection. The P.I. of 60 was essentially the same as that obtained

TABLE VII

Susceptibility of the MB III Cell to Infection with R. tsutsugamushi (Karp Strain) as Influenced by Preincubation in the Presence or Absence of L-*Glutamic Acid*

Tube	Treatment of MB III cells	Additions to cells	Penetration index after incubation at 37°C.
			120 min.
a	None*	Rickettsiae + BSS	51
b	None	" + L-glutamic acid‡	139
c	120 min. at 37°C. in BSS	" + BSS	60
d	120 min. at 37°C. in BSS	" + L-glutamic acid	144
e	120 min. at 37°C. in L-Glutamic acid	" + BSS	143

* Cells washed free of growth medium and suspended in BSS immediately prior to the addition of a freshly prepared rickettsial inoculum.

‡ L-Glutamic acid, when present, was at a final concentration of 0.006 M in BSS.

with the untreated control tube (tube *a*). The addition of glutamic acid after preincubation, and concomitant with the rickettsial inoculum (tube *d*) did not affect the P.I. when compared with the untreated glutamate control (tube *b*). Similarly, the presence of glutamic acid during the preincubation period did not influence the number of organisms invading the cell. It is of interest that glucose in the BSS serves as an available source of energy for the MB III cell, whereas the rickettsiae are unable to utilize this compound.

The Effect of Temperature on the Action of L-Glutamic Acid.—Although glutamic acid was capable of increasing penetration, presumably through an effect on the rickettsiae, the mechanisms by which this was brought about were not clear. The inability of D-glutamic acid to substitute in the penetration system suggested a metabolic role for the levo isomer rather than an effect as a non-specific adsorption cofactor. The following experiments demonstrated that the action of L-glutamic acid was suppressed by incubation at low temperature.

Four roller tubes containing the identical concentrations of rickettsiae, MB III cells and L-glutamic acid (0.006 M) were prepared. Tube *a* was maintained at 37°C. for the entire 120 minute incubation period while *b* remained at 4°C. Tube *c* was incubated at 4°C. for 30 minutes and then placed at 37°C. for 90 minutes. Tube *d* was maintained at 4°C. for 60 minutes and then at 37°C. for a similar period of time. Smears were prepared after 30, 60, and 120 minutes of incubation. A set of control tubes in which BSS alone was the suspending medium also was prepared and incubated along with those containing glutamate.

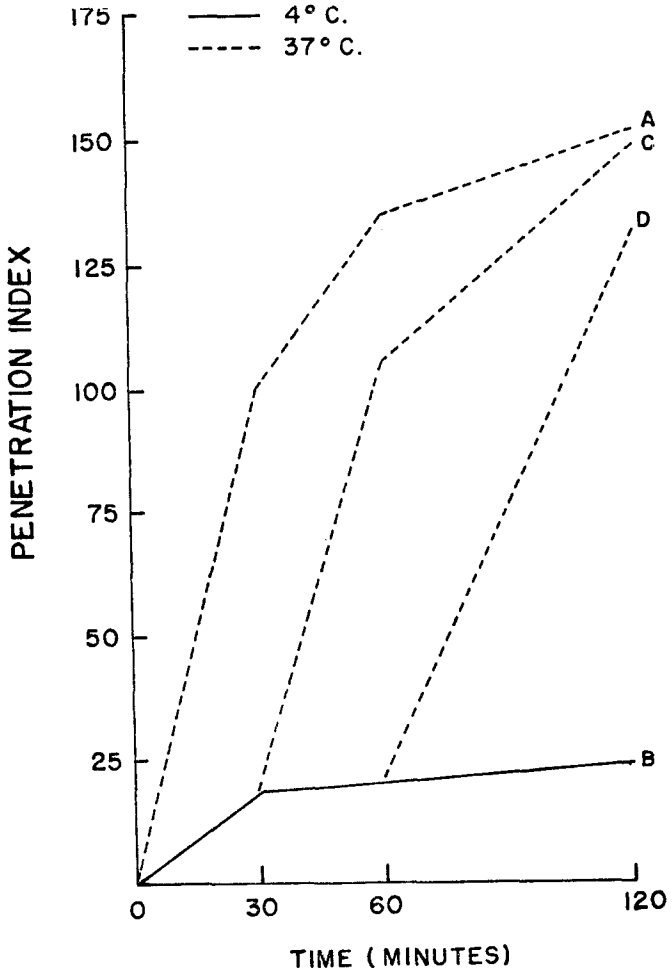

FIG. 3. The effect of temperature on the enhancing action of L-glutamic acid

Fig. 3 presents graphically the results obtained when BSS containing glutamic acid was employed. Line *A* shows the P.I.'s obtained when the tubes were incubated at 37°C. throughout the experiment. Line *B* represents the cultures kept at 4°C. for the entire period. Penetration was minimal and the pres-

ence of glutamate did not alter the P.I. at this temperature. On the other hand, smears prepared from cultures which first had been held at 4°C. for 30 and 60 minutes (lines *C* and *D*, respectively) and then were placed at 37°C. for the remainder of the experiment exhibited a marked increase in the numbers of intracellular rickettsiae when compared with the 4°C. control. Although the effects of low temperature are complex, it appears that glutamate increases penetration only at the temperatures at which it can be maximally oxidized and serve as a source of rickettsial energy.

TABLE VIII

Effect of Metabolic Inhibitors on the Penetration of R. tsutsugamushi (Karp Strain) into MB III cells in the Presence and Absence of L-*Glutamic Acid*

Medium	Inhibitor	Final concentration in BSS	Penetration index after incubation at 37°C.		Per cent of control	
			30 min.	120 min.	30 min.	120 min.
		M				
BSS	DNP*	0.0001	2	4	4‡	7
	Arsenite	0.001	19	20	39	34
	Cyanide	0.001	11	10	22	17
	Azide	0.0013	18	18	37	31
BSS + L-glutamic acid (0.006 M)	DNP	0.0001	30	32	30§	23
	Arsenite	0.001	24	41	24	31
	Cyanide	0.001	15	19	15	14
	Azide	0.0013	12	22	12	16

* 2,4-Dinitrophenol.

‡ Values calculated from the BSS control of each experiment.

§ Values calculated from the L-glutamic acid control of each experiment.

The Effect of Metabolic Inhibitors on the Action of L-Glutamic Acid.—The studies of Wisseman *et al.* (23) have shown that various metabolic inhibitors interfere with the normal pathway of glutamate oxidation by *R. mooseri*. Consequently, experiments were done to determine the effect of these inhibitors on the penetration of *R. tsutsugamushi* in the presence and absence of glutamic acid.

Table VIII presents the results obtained when 2,4-dinitrophenol, arsenite, cyanide, and azide were incorporated in the system. The upper portion of the table shows that the inhibitors caused a marked reduction in the P.I. when the rickettsiae and cells were suspended in BSS alone, with 2,4-dinitrophenol producing the greatest suppressive effect. The lower section of the table shows that when the inhibitors and glutamic acid were added simultaneously there was a similar reduction in penetration. Thus, it appears that the inhibitors block the enhancing effect of glutamate in the penetration process.

The metabolic inhibitors did not exhibit an *in vitro* rickettsiocidal action, nor

did they produce any irreversible alteration in the susceptibility of the MB III cell.

Standard suspensions of rickettsiae exposed for 40 minutes to the concentrations of inhibitor indicated in Table VIII caused no decrease in infectivity as determined by titrations in mice. Likewise, suspensions of MB III cells which were incubated for 120 minutes in the presence of each inhibitor, washed once with BSS, and then exposed to standard infectious inocula yielded P.I.'s identical with the untreated controls.

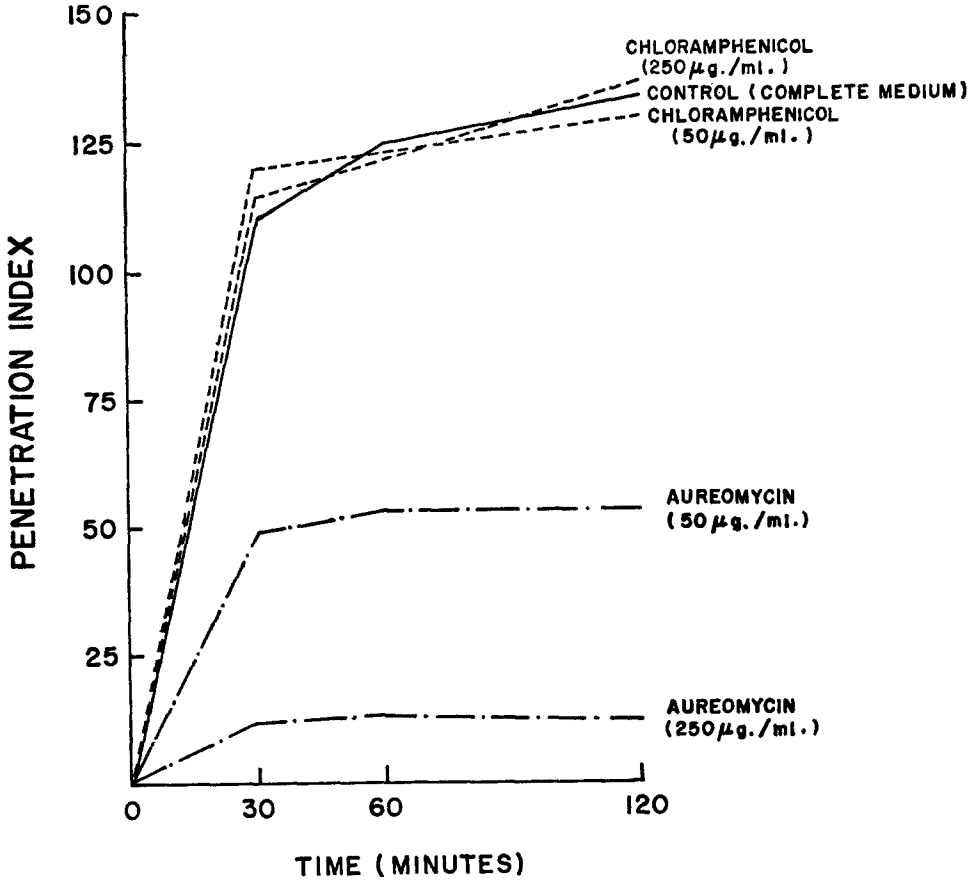

FIG. 4. The effect of aureomycin and chloramphenicol on penetration

The Effect of Aureomycin and Chloramphenicol on Penetration.—Previous studies on the effects of broad spectrum antibiotics on the typhus group of rickettsiae revealed the *in vitro* rickettsiocidal action of aureomycin in concentrations exceeding 300 μg./ml. (24). Evidence supporting this view was concerned with the ability of this drug to depress toxicity, infectivity, hemolysis,

and the oxidation of glutamic acid by the organisms. In contrast, chloramphenicol was inactive at similar concentrations.

No influence on penetration was noted when chloramphenicol was incorporated in the complete medium at concentrations of 50 and 250 μg./ml. (Fig. 4). Aureomycin, however, at similar concentrations produced a marked depression of the P.I., which was most striking at 250 μg./ml. Since the effect of this antibiotic could possibly have been on both the rickettsiae and the host cell, further studies were conducted to differentiate this site of action.

Titrations in mice of standard suspensions of *R. tsutsugamushi* incubated at 37°C. for 30 minutes in the presence of 250 μg./ml. of aureomycin showed a 1,000-fold reduction in infectivity as compared with the complete medium control. MB III cells, however, when incubated for periods up to 120 minutes with the same concentration of drug, washed, and then exposed to an infectious inoculum became infected to the same extent as the untreated control. Thus, it appears that the action of aureomycin in depressing the P.I. is most likely related to the rickettsiocidal action of the drug.

DISCUSSION

In general, those factors which affect the viability of *R. tsutsugamushi* (Karp strain), as measured by mouse titration, also influence the number of rickettsiae which invade the MB III cell. A strict correlation, however, between mouse infectivity and penetration index is not feasible. In part, this may be related to the relative insensitivity of the animal assay in which 4- to 8-fold differences in the number of infectious particles could escape detection, while the P.I. would show an appreciable variation. Furthermore, there are probably many other variables in the mouse assay which are not present in an *in vitro* cell culture system.

The activity of many of the factors which enhance penetration appears to be related to their ability to maintain the infectivity of the rickettsiae during the course of the penetration experiment. Such factors include glutamic acid and related metabolites, all of which have been shown to be actively oxidized by purified suspensions of rickettsiae. This correlation between *in vitro* metabolic activities of purified rickettsial suspensions and the factors affecting penetration is further emphasized by the failure of a variety of amino acids and Krebs' cycle intermediates to exhibit appreciable activity in either assay system. It must be pointed out, however, that no experimental data are currently available on the metabolic properties of *R. tsutsugamushi*, and the analogies between those factors affecting penetration and biochemical properties of the isolated organisms are entirely drawn from studies on *R. mooseri* (5, 23), *R. prowazekii* (4, 7, 17), and *R. rickettsii* (25).

There are, however, other elements related to the maintenance of rickettsial viability which do not serve any obvious metabolic role. Two such examples

are serum albumin and the divalent cations. In the case of albumin this may be related to the general protective effects of proteins on the stability of other microorganisms (26), which would allow the normal metabolic functioning of the rickettsiae in a non-cellular environment. On the other hand, the effects of the divalent cations in the penetration system may not be associated with the maintenance of rickettsial metabolism *per se*, as suggested by the non-specificity of the requirements, but with an initial electrostatic binding of the parasite to the MB III cell. Certain analogies exist with other host-parasite interactions in which this is the case (1). Because of the design of the penetration system as employed in these studies such adsorptive phenomena could not be evaluated.

The ability of enzymatic inhibitors to depress the penetration of *R. tsutsugamushi* roughly parallels their action both on the oxidation of glutamic acid and on the hemolysis of red cells as performed by purified suspensions of the typhus group. One exception is the ability of 2,4-dinitrophenol to block penetration, both in the presence and absence of glutamic acid, whereas no such effect is noted on oxygen consumption (23). The relationship between this finding and the recently discovered role of adenosinetriphosphate in rickettsial metabolism is not clear. Another discrepancy is found with sodium azide which depresses the oxidation of glutamic acid and the hemolysis of red cells (8, 23) to a small extent but is a potent inhibitor of penetration. Although no strict correlation between biochemical properties and penetration is possible at this time, it nevertheless seems likely that the biochemical integrity of the parasite is in some fashion related to its ability to penetrate the host cell.

The role of the MB III cell in the penetration process is poorly understood but seemingly contributes less actively to the initial stages of infection than do the rickettsiae themselves. Studies in which the strain L cell was employed gave qualitatively similar results and support this contention. It would seem, however, that the contribution of the host cell becomes more important for the subsequent multiplication of the rickettsiae by supplying a proper physicochemical environment. Although large numbers of viable rickettsiae are able to infect the MB III cell, the capabilities of this strain to become infected with a number of bacteria is quite limited. Indeed, the addition of high multiplicities (100 bacteria/cell) of both *Escherichia coli* and *Micrococcus pyogenes* var. *aureus*, under similar conditions, resulted in only a small percentage of cells which contained intracellular organisms. This was true whether the bacteria were viable or had been inactivated. Such results suggest a decided difference in the interaction of the MB III cell with rickettsiae and bacteria.

SUMMARY

A system has been described in which the penetration of *Rickettsia tsutsugamushi* (Karp strain) into tissue culture cells can be quantitated, and the factors affecting this process studied. The results indicated that rickettsial penetra-

tion *in vitro* depended largely on the viability of the organisms. Certain components of the fluid environment such as the divalent cations and protein were found to be of importance. The temperature dependence of the penetration process was found to vary with the nature of the suspending medium. A number of compounds related to L-glutamic acid enhanced penetration, whereas metabolic inhibitors depressed this process. Aureomycin at concentrations between 50 and 250 μg./ml. inhibited the penetration of rickettsiae while chloramphenicol at similar concentrations was ineffective. The results are discussed in terms of the biological and biochemical properties of this group of agents.

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