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Effects of Various Suspending Media on Plaque Formation by Rickettsiae in Tissue Culture

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Effects of some media used for suspending rickettsiae during purification, for metabolic studies, and in titrations of infectious rickettsiae were examined with respect to the plaque-forming ability of *Rickettsia rickettsi* and *R. typhi* in primary chicken embryo tissue cultures and the infectivity of *R. typhi* in mice. Brain heart infusion broth (BHI) was found superior to all other media tested in preventing both a significant decrease in plaque-forming units (PFU) and a delay in plaque formation. Skim milk, egg yolk, and some metabolic media were effective in maintaining PFU at 0 C, but did not prevent a significant delay in plaque formation. However, infectivity of *R. typhi* for tissue culture and mice was markedly decreased when suspended in metabolic media at 26 C. Addition of BHI to the routine tissue culture overlay reversed the deleterious effects of sucrose-phosphate solutions. The effects of Mg^{2+}, Mn^{2+}, K^{+}, Na^{+}, sucrose, and glutamate were also examined. No significant differences were observed between *R. rickettsi* and *R. typhi* in their responses to different media. The results of this study suggest the necessity for a reappraisal of previous studies of metabolism and infectivity of rickettsiae in these media.

It has been recognized that most species of pathogenic rickettsiae (possibly excluding *Coxiella burneti*) rapidly lose viability in many media, even at 0 C. It is desirable to use media as diluents for purification, and for measurement of infectious and metabolic activity, which preserve the normal biological activity of rickettsiae. Skim milk and 20% suspension of normal chicken embryo yolk sacs in nutrient broth were previously shown to favor rickettsial survival (2). However, these turbid media are not desirable for purification of viable rickettsiae. Under certain conditions rickettsiae have also been shown to remain fairly stable in both serum albumin media and sucrose-phosphate-glutamate (SPG) media (8), as measured by animal and embryonated chicken egg assays, or in K36 + bovine plasma albumin (BPA) media (17, 20), as measured by metabolic activity.

This report summarizes the results of an examination of the effects of some rickettsial media, previously used by other investigators, on the plaque-forming ability of *Rickettsia rickettsi* and *R. typhi*. It is a continuation of a previous study (21) in which brain heart infusion broth (BHI) was found to be superior to sucrose-phosphate (SP25) medium (20) and SPG in maintaining rickettsial infectivity.

MATERIALS AND METHODS

Rickettsial seeds. Seeds were prepared as previously described (21). The rickettsiae employed were *R. typhi* Wilmington strain, with a history of 12 passages in embryonated eggs, 15 in guinea pigs, and then 3 in embryonated eggs (12EP/15GP/3EP); and *R. rickettsi* "R" strain (53EP).

Cell culture and plaque procedure. As previously described (21), primary chicken embryo (CE) tissue cultures, prepared 24 hr earlier, were inoculated with 0.1 ml of rickettsial suspensions and incubated for 15 min at room temperature. A nutrient overlay was then applied, the tissue cultures were incubated at 32 C, and the plaque-forming units (PFU) were determined after cell sheets had been stained with a second overlay containing neutral red.

Mouse infectious end points. The 50% infectious dose per milliliter (ID_{50}) in mice was determined by serologic conversion, measured by complement fixation (12) and microagglutination (13) tests with homologous antigen, as previously described (21). Mouse ID_{50} values were computed by the Spearman-Kärber method (11).

Preparation of media. All solutions were prepared in double glass-distilled water with reagent grade commercial chemicals. Abbreviations and compositions of media are listed in Table 1. The metabolic media listed were used by various investigators in the measurement of rickettsial respiration. Skim milk and BHI were

TABLE 1. *Moles per liter chemical composition of media tested*[a]

Medium	Sucrose	KCl	NaCl	KH_2PO_4	K_2HPO_4	NaH_2PO_4	Na_2HPO_4	K^+ glutamate	$MgCl_2$	$MnCl_2$	NAD	BPA	pH
1. PBS			0.072	0.013			0.054						7.35
2. KCl-K^+ phosphate		0.13		0.02									7.0
3. KCl-K^+ phosphate-Mg^{2+}, Mn^{2+}		0.13		0.02					0.0016	0.00026			7.0
4. NaCl-Na^+ phosphate			0.13			0.02							7.0
5. NaCl-Na^+ phosphate-Mg^{2+}, Mn^{2+}			0.13			0.02			0.0016	0.00026			7.0
6. Solution X	0.20	0.10		0.02									7.25
7. SP25	0.25			0.0167	0.0333								7.2
8. SPG	0.215			0.0038	0.0058			0.0048					7.2
9. K7G		0.122	0.0074	0.0041			0.0078	0.0049					7.0
10. K36 + BPA		0.10	0.015	0.05								3 mg/ml	7.0
11. Wisseman et al. metabolic		0.125	0.02	0.0012			0.0106	0.05	0.0016	0.00026			7.4
12. Solution X metabolic	0.20	0.10		0.02				0.0072	0.0046	0.00055	0.00059		7.25
13. Allen et al. metabolic	0.09	0.048		0.01				0.0125	0.001	0.00033			7.4
14. K36 metabolic		0.10	0.015	0.05				0.0083	0.0025	0.0005		3 mg/ml	7.0
15. Skim milk													7.3
16. BHI													7.2

[a] Abbreviations and references: PBS, phosphate-buffered saline (19); solution X and solution X metabolic medium (16); SP25, sucrose-phosphate solution (20); SPG, sucrose-phosphate-glutamate solution (8); K7G (8); K36 + BPA (bovine plasma albumin; Metrix, Armour Pharmaceutical Co., Chicago, Ill.) (20) and K36 metabolic medium (17, 20); Wisseman et al. metabolic medium (22, 23); Allen et al. metabolic medium (1); skim milk (Difco), added 100 g of powdered skim milk to 1 liter of distilled water; BHI, brain heart infusion broth (Difco) made by adding 37 g of BHI to 1 liter of distilled water; NAD, nicotinamide adenine dinucleotide.

sterilized by autoclaving. All other media were sterilized by passage through Falcon No. 7103 disposable filters (Falcon Plastics, Los Angeles, Calif.). Because some filters contain detergents which may be harmful to biological systems (10), the filters were first rinsed with double glass-distilled water at about 90 C, and then all media were vacuum-filtered at 0 C.

RESULTS

Effect of suspending media at 0 C on rickettsial PFU and plaque morphology. *R. typhi* and *R. rickettsi* were diluted in various ice-cold media and assayed in CE tissue cultures after 0 min (15 min allowed to make dilutions and immediately inoculate) and 90 min on ice (Table 2). With both organisms, the simple salt-phosphate (Table 2, no. 1–5) and sucrose media (Table 2, no. 6–8) were least effective for maintaining PFU. The salt-phosphate media were also most detrimental to plaque size and clarity (Fig. 1E). K^+ salt-phosphate media were slightly better in maintaining PFU than those containing Na^+. Addition of Mg^{2+} and Mn^{2+} to salt-phosphate solutions resulted in very slight improvement in PFU values with either organism, but addition of glutamate to a sucrose medium (as in SPG) significantly increased PFU over a similar medium without glutamate (SP25). Use of K7G (Table 2, no. 9) produced an improvement in maintaining PFU at 0 min over both salt-phosphate and sucrose media. The metabolic media (Table 2, no. 11–14) were much better for maintaining PFU, the least effective in this group being that which contained sucrose (solution X metabolic medium). But still they adversely affected eventual plaque size and clarity (Fig. 1D) as compared to BHI (Fig. 1A). Only in K36 + BPA, K36 metabolic medium, and skim milk was the PFU value maintained near that of BHI for 90 min for both organisms. However, even with these media the plaque size and clarity 9 days postinoculation (PI) were reduced markedly (Fig. 1C and D) when compared to plaques produced 9 days PI by rickettsiae suspended in BHI (1A). In fact, plaques produced 9 days PI with any other medium tested were no larger than those produced 5 days PI by *R. rickettsi* suspended in BHI (Fig. 1B). Even then they were more poorly defined than those produced by BHI suspensions. In each medium tested, changes in PFU and plaque morphology were similar for *R. typhi* and *R. rickettsi*.

Effect of normal yolk. Normal yolk which had been obtained from 10-day-old embryonated chicken eggs and shell-frozen as a 50% suspension in SP25, as the rickettsial seed was prepared, was diluted (v/v) to concentrations of 1%, 0.1%, and 0.01% yolk in SP25 and used as an ice-cold diluent for *R. rickettsi* (Table 3). Only 1% yolk in SP25 medium maintained PFU identical to that for rickettsiae suspended in BHI. However, *R. rickettsi* suspended in SP25 containing 1% yolk produced plaques only 0.7 mm in diameter compared to the 2.0 mm diameter plaques produced by BHI suspensions. Differences in plaque morphology were not noted for SP25 and yolk-SP25

TABLE 2. *Effect of various media[a] on plaque-forming units per milliliter for Rickettsia typhi and R. rickettsi*

Medium	*R. typhi*		*R. rickettsi*	
	0 min[b] at 0 C	90 min at 0 C	0 min at 0 C	90 min at 0 C
1. PBS	16×10^6	2×10^6	1×10^6	1×10^6
2. KCl-K^+ phosphate	18×10^6	3×10^6	4×10^6	2×10^6
3. KCl-K^+ phosphate-Mg^{2+}, Mn^{2+}	29×10^6	12×10^6	11×10^6	2×10^6
4. NaCl-Na^+ phosphate	3×10^6	2×10^6	2×10^6	1×10^6
5. NaCl-Na^+ phosphate-Mg^{2+}, Mn^{2+}	4×10^6	3×10^6	4×10^6	2×10^6
6. Solution X	9×10^6	1×10^6	3×10^6	1×10^6
7. SP25	4×10^6	1×10^6	9×10^6	3×10^6
8. SPG	11×10^6	5×10^6	25×10^6	8×10^6
9. K7G	63×10^6	1×10^6	54×10^6	1×10^6
10. K36 + BPA	250×10^6	170×10^6	142×10^6	32×10^6
11. Wisseman et al. metabolic	170×10^6	46×10^6	138×10^6	3×10^6
12. Solution X metabolic	53×10^6	21×10^6	54×10^6	21×10^6
13. Allen et al. metabolic	180×10^6	51×10^6	108×10^6	4×10^6
14. K36 metabolic	290×10^6	230×10^6	128×10^6	78×10^6
15. Skim milk	230×10^6	200×10^6	120×10^6	110×10^6
16. BHI	322×10^6	298×10^6	130×10^6	112×10^6

[a] See Table 1 for preparation and abbreviations for all media.
[b] Zero minutes equals the approximate 15 min to make dilutions and inoculate tissue cultures.

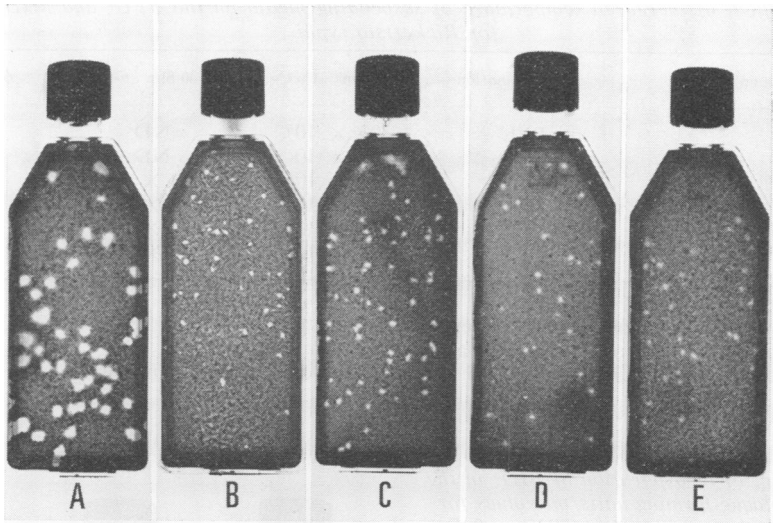

FIG. 1. *Typical effects of suspending media on plaque morphology of Rickettsia rickettsi. The rickettsiae were held at 0 C in the specified medium until inoculation of primary chicken embryo tissue cultures. A, Brain heart infusion broth (BHI) at 9 days postinoculation (PI); B, BHI, 5 days PI; C, 10% skim milk, 9 days PI; D, sucrose-phosphate (or any medium in the group numbered 7 to 14, Table 2), 9 days PI; E, phosphate-buffered saline (or any medium in the group numbered 1 to 5, Table 2), 9 days PI.*

media. Similar results were obtained with *R. typhi*.

Effects of increased temperature of the metabolic media. *R. typhi* was suspended in either K36 metabolic or Wisseman et al. metabolic medium at room temperature (26 C) and on ice (0 C) for up to 60 min and then inoculated into tissue cultures and mice to determine effects of temperature on PFU and mouse ID_{50} values (Table 4) and plaque morphology. *R. typhi* suspended in BHI at 0 C was tested for stability at 0 min only, because previous (21) and subsequent observations indicated that this organism was completely stable in BHI. Numbers of PFU, as well as plaque size and clarity, decreased when the metabolic media were used at 0 C (as reported in a preceding section), and the deleterious effect was even more dramatic when the media were at 26 C. The deleterious effect of both metabolic media at 26 C on the mouse ID_{50} was even greater than the effect on the PFU value.

Addition of BHI to the agarose overlay. Identical suspensions of *R. rickettsi* in BHI and SP25 at 0 C were inoculated onto CE tissue cultures. Half of each group of cultures were then overlaid with standard agarose-medium 199-5% fetal calf serum (21), and the other half with overlay-5% BHI (95 ml of standard overlay + 5 ml of standard BHI). The addition of this small amount of BHI to the overlay not only increased the PFU for the rickettsiae suspended in SP25 (Table 5), but also markedly restored the plaque morphology (Fig. 2) to very nearly that of rickettsiae suspended in BHI. The addition of BHI to the overlay did not affect PFU or plaque morphology of rickettsiae suspended in BHI. (Studies in this laboratory have shown that medium 199 + 5% fetal calf serum used as a diluent at 0 C for both *R. rickettsi* and *R. typhi* will also significantly decrease PFU and plaque size and clarity.)

TABLE 3. *Effect of yolk in SP25[a] diluent on plaque-forming units per milliliter for Rickettsia rickettsi*

Diluent	0 min[b] at 0 C	90 min at 0 C
BHI[c]	126×10^6	118×10^6
1% Yolk in SP25	128×10^6	128×10^6
0.1% Yolk in SP25	122×10^6	52×10^6
0.01% Yolk in SP25	54×10^6	24×10^6
SP25	20×10^6	5×10^6

[a] Sucrose-phosphate solution.
[b] Zero minutes equals the approximate 15 min to make dilutions and inoculate tissue cultures.
[c] Brain heart infusion broth.

Autoclaved versus filter-sterilized BHI. Duplicate portions of BHI solution were sterilized by autoclaving or by passage through a Falcon no. 7103 disposable filter. The method of sterilization had no effect on PFU or plaque morphology with either *R. typhi* or *R. rickettsi* suspended in BHI at 0 C.

TABLE 4. *Effect of increased temperature of suspending media on the PFU[a] and MID_{50}[b] values for Rickettsia typhi*

Medium[c]	Determination	0 min[d] at 0 C	60 min at 0 C	60 min at 26 C
BHI	PFU	300×10^6	ND[e]	ND
	MID_{50}	250×10^7	ND	ND
K36 metabolic	PFU	270×10^6	230×10^6	10×10^6
	MID_{50}	200×10^7	130×10^7	1.6×10^7
Wisseman et al. metabolic	PFU	170×10^6	50×10^6	15×10^6
	MID_{50}	130×10^7	64×10^7	0.4×10^7

[a] Plaque forming units per milliliter.
[b] Mouse 50% infectious dose per milliliter.
[c] See Table 1 for preparation and abbreviations for all media.
[d] Zero minutes equals the approximate 15 min to make dilutions and inoculate tissue cultures and mice.
[e] Not done.

TABLE 5. *Effect of incorporation of BHI[a] in the overlay on plaque-forming units/ml values for Rickettsia rickettsi diluted in SP25[b] at 0 C*

Overlay	Diluent	
	BHI	SP25
Routine[c]	128×10^6	15×10^6
Routine with 5% BHI	122×10^6	52×10^6

[a] Brain heart infusion broth.
[b] Sucrose-phosphate solution.
[c] Routine overlay was 0.5% agarose-medium 199 + 5% fetal calf serum.

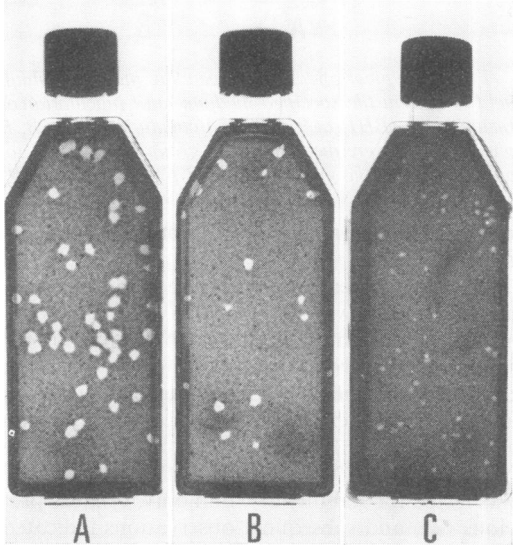

FIG. 2. *Effect on morphology of Rickettsia rickettsi plaques induced by 5% (v/v) brain heart infusion (BHI) in the nutrient overlay. A, BHI diluent with or without BHI overlay; B, sucrose-phosphate (SP25) diluent (Table 2) with 5% BHI in overlay; C, SP25 diluent without BHI in overlay. Nine days postinoculation.*

DISCUSSION

BHI at 0 C appeared to be the best diluent for maintaining rickettsial infectivity, as measured by PFU values and plaque morphology. Only skim milk, K36 + BPA, and K36 metabolic medium at 0 C were able to maintain PFU values for rickettsiae near that of rickettsiae in BHI. However, even these media caused a reduction in plaque size and clarity. This may indicate that those organisms which eventually produced plaques had sustained damage which markedly delayed plaque growth by at least 4 days (Fig. 1), although the possibility that the diluents also affected the host cells has not been ruled out. In all the media tested there may be a lack of factors for maintaining rickettsial infectivity rather than a toxic factor common to all.

Sucrose appeared to be ineffective in stabilization of rickettsial infectivity. The deleterious effect of sucrose was reported (7) when oxygen utilization by rickettsiae was decreased by 20% in metabolic studies done in SPG as compared to those done in albumin-glutamate-KCl-NaCl solution. Later it was observed (4) that rickettsiae purified in a sucrose-phosphate solution showed very poor stability, as measured by hemolytic and toxic activities, when compared to preparations purified in a salt-phosphate solution. Similar differences in infectivity were noted between SPG (sucrose) and K7G (salt) media (Table 2). Even though the presence of sucrose in SPG may not have been particularly effective, the presence of glutamate (as in SPG versus SP25) (Table 2) had, as has been shown previously (4, 8, 9, 17, 21, 23), a marked stabilizing influence on rickettsiae.

Stoenner et al. (19) reported that *R. rickettsi* suspended in SPG and inoculated into embryonated chicken eggs allowed the eggs to survive longer and resulted in poorer antigen toxin than eggs inoculated with identical suspension of *R. rickettsi* in 0.1 M phosphate-buffered saline. In contrast, we found that SPG was much better in maintaining infectivity of *R. rickettsi* in tissue cultures than was phosphate-buffered saline. The addition of Mg^{2+} or Mn^{2+} to a simple salt-phosphate solution did not significantly improve rickettsial PFU, in contrast to earlier findings in egg and mouse assay systems (8). However, oxygen utilization by rickettsiae in the more complete metabolic media is increased significantly by Mg^{2+} and Mn^{2+} (4, 7, 23). Finally, a medium high in K^+ was generally more favorable for maintaining rickettsial infectivity than one high in Na^+. Similar effects have been reported by others (8). The effect is perhaps related to the high K^+ content of host cell interiors in which rickettsiae are obligatory parasites.

Although rickettsial infectivity is lost much more rapidly than respiratory activity (1, 17), the results of previous studies of rickettsial metabolism may have been significantly influenced by the medium in which they were suspended. The capacity of rickettsiae to utilize oxygen (7, 9, 16, 17, 20, 22, 23) is generally lower than that recorded for bacteria and yeast under optimal conditions, and oxygen utilization by typhus rickettsiae is decreased 20% in SPG (7). In addition, rickettsiae used for studies of respiratory activity were purified (yolk removed) for several hours near 0 C in media such as SPG (22, 23), K7G and SPG (1, 7, 9), solution X (16), and SP25 (17, 20), all of which we have found to reduce infectivity of rickettsiae (Table 2), even when some protective yolk is present (Table 3) (8). Rees and Weiss (17) reported a 75 to 96% decrease in infectivity of rickettsiae purified in SP25. This decrease may have been due to loss of rickettsiae during purification, or it may have been due to inactivation of the rickettsiae by the SP25 medium. Also, Myers et al. (15) have shown that permeability of rickettsiae is greatly altered by purification in SPG. Finally, we have found that an increase in the temperature of metabolic media (from 0 to 26 C) in which the rickettsiae were suspended resulted in marked decreases in PFU and mouse ID_{50} values. Thus, the combination of purifying rickettsiae in various deleterious media and then measuring their metabolic activity in other such media at temperatures of 30 to 34 C may have contributed to decreases in oxygen utilization. It is possible that the changes in rickettsial membrane permeability reported by Myers et al. (15) also occurred with the suspending media which we have examined. Such changes may be the basis for decreases in rickettsial infectivity which we have measured by PFU values, plaque morphology, and mouse ID_{50} values.

Whatever the reasons for the adverse effect of the suspending media tested here, the adverse effect of SP25 on plaque size and PFU was readily reversed by the addition of small amounts of BHI to the overlay (Table 5). The rickettsiae in SP25 were not dead, since it appears that BHI supplied a factor not present in either SP25 or the nutrient overlay, which restored the ability of rickettsiae to enter or multiply in the CE cells, or both. (As reported in a previous section, the medium 199 + 5% fetal calf serum of the nutrient overlay is, in fact, deleterious to rickettsial PFU and plaque size and clarity.) This factor (or factors) was heat stable, because autoclaving BHI before use had no effect on plaque morphology or PFU values. Reversible reactivation of rickettsiae has been accomplished by various combinations of Mn^{2+}, Mg^{2+}, nicotinamide adenine dinucleotide (NAD) and/or coenzyme A (3, 5, 6), glutamate, pyruvate or adenosine triphosphate (5), and glutathione (17). Reversal of "avirulent" rickettsiae to "virulent" by NAD, coenzyme A, or *p*-aminobenzoic acid (14), or by feeding ticks infected with rickettsiae a blood meal (18) has also been noted. Whether any of these factors, or the factors in BHI, allows the rickettsiae to enter the cell, multiply in the cell once they are there, or both, is not known.

It is important to note that a decrease in PFU caused by the use of deficient suspending media, or by raising the temperature of these media, is reflected by a corresponding decrease in the mouse ID_{50} (Table 4) (21). This indicates that the PFU value obtained from assay of infectious rickettsiae in CE tissue cultures is a valid and sensitive biological indicator of infectious rickettsiae in other assay systems. Because it has been shown by others (1, 17) that decreases in infectivity of rickettsiae were associated with decreases in metabolic activity, changes in PFU values may, therefore, reflect changes in metabolic capabilities of rickettsiae.

All media tested, with the possible exception of BHI, apparently lack some factor or factors necessary for normal biological activity of rickettsiae. Because the infectious titers of rickettsiae in the various media decline even at 0 C, different laboratories may obtain different infectious end points for identical seed materials. In most cases major decreases in infectious end points occurred rapidly, within 15 min at 0 C. Previous observers, using less sensitive assay systems and without the benefit of BHI, would have been unable to demonstrate these sudden decreases in infectivity of rick-

ettsiae suspended in deleterious media. The results of this study suggest that a re-examination of some of the biological properties of rickettsiae, which were determined in suspending media now shown to be deficient, might result in more realistic estimates of rickettsial capabilities.

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