GROWTH OF *RICKETTSIA PROWAZEKI* IN IRRADIATED MONOLAYER CULTURES OF CHICK EMBRYO ENTODERMAL CELLS[1]

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This paper is concerned with the growth of *Rickettsia prowazeki* in monolayer cultures of chick embryo entodermal cells by a method similar to that employed by Weiss and Pietryk (1956) for *Coxiella burnetii*. In an attempt to enhance the growth of the rickettsiae, the host cells were exposed to Co^{60} γ-rays prior to inoculation. This procedure was prompted by the demonstration of the enhancing effect of X-irradiation on the growth of rickettsiae in experimental animals and in the yolk sac of chick embryos by several investigators (Zinsser and Castaneda, 1932; Liu *et al.*, 1941; Kawamura and Shimizu, 1955; and Greiff *et al.*, 1953, 1955, 1957*a*, *b*). It was also suggested by the results of Puck and Marcus (1956) and Cieciura *et al.* (1957), who showed that X-rays elicited the transformation of some mammalian cells *in vitro* into giant cells, which were highly susceptible to virus infection and produced high virus titers.

A true enhancement of rickettsial growth was not obtained, but a number of observations on the effect of γ-rays on chick embryo entodermal cells and on the growth of *R. prowazeki* in irradiated and unirradiated cultures were made and are here presented.[2]

MATERIALS AND METHODS

Rickettsiae. Two Madrid E strains (Perez Gallardo, and Fox, 1948) of *Rickettsia prowazeki*, parent and *p*-aminobenzoic acid-resistant mutant (Weiss *et al.*, 1957), were used. Since the two strains appeared to grow equally well in tissue culture in preliminary experiments, the work was continued with only the drug resistant strain.

Pools of rickettsiae were prepared from infected chick embryo yolk sacs suspended in the isotonic solution containing sucrose, phosphates, and glutamate of Bovarnick *et al.* (1950) and partially purified by a previously described simplified method (Weiss *et al.*, 1957). The procedure included homogenization of the yolk sacs, treatment with trypsin, high-speed centrifugation, and precipitation of yolk sac constituents in sucrose-phosphate-glutamate solution containing 6 per cent bovine plasma albumin. The precipitate was eliminated by low-speed centrifugation and the rickettsiae remained suspended in a volume of albumin sucrose-phosphate-glutamate solution which corresponded to the weight of the yolk sacs. The partially purified rickettsial suspensions were stored in the dry ice cabinet until needed.

Tissue cultures. The techniques for the explantation of the avascular membranes of 4-day-old embryos and preparation of monolayer entodermal cell cultures were similar to those previously described (Weiss and Huang, 1954; Weiss and Pietryk, 1956), but the following two simplifications were introduced: (1) Test tubes, 16 by 150 mm, with one flat side just fitting an 11 by 22 mm cover slip, were used instead of Porter flasks; (2) The explants were not embedded in plasma, but merely placed in 0.6 ml of medium on the cover slip. Most of the explants were found adhering to the cover slip at 24 hr and beginning to form a monolayer, which was ready for use on the 3rd or 4th day. In a few cases other types of tubes or Erlenmeyer flasks with or without cover slips were used. The medium consisted of serum, 25 per cent, obtained from roosters maintained on an antibiotic-free diet, and Hanks' balanced salt solution (Hanks and Wallace, 1949).

The cultures were usually infected on the 3rd day after explantation by removing the old medium and replacing it with new medium of the same composition, but containing 1 to 2 per cent partially purified rickettsial suspension. The cul-

[1] The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

[2] Some of this material was presented at the Eighth Annual Meeting of the Tissue Culture Association in Baltimore, 1957.

tures were incubated at 36 C before and at 35 C after inoculation.

For microscopic observations the cultures on cover slips were fixed in absolute methanol and stained by the May-Gruenwald-Giemsa procedure.

Rickettsial titrations. The extent of rickettsial growth was determined by the microscopic examination of the cultures and by the titration of their nutrient fluids in eggs. In most cases 3 cultures were used per determination, but in many cases this number was increased to 4 and up to 9.

The rickettsial titers of pooled nutrient fluids were determined by the single dilution method as in previous experiments (Weiss *et al.*, 1957). Each fluid to be titrated was diluted 1:10 with the sucrose-phosphate-glutamate solution and injected, via yolk sac, into 15 chick embryos, 7-day-old, 0.5 ml per embryo, and the mean survival time of the embryos, exclusive of nonspecific deaths, was determined. The rickettsial titers were determined from the logarithms of the mean survival time, as in the previous paper (Weiss *et al.*, 1957), and expressed as the number of infectious rickettsiae per culture.

Irradiation. The Co^{60} irradiator was described in detail by Morgan and Ellinger (1956). It consists essentially of 60 individual 25- to 50-curie sources, positioned in such a way as to produce a uniformly distributed γ-ray flux inside a 9 by 30 in cylinder. The intensity of the γ-radiation was approximately 455 r/air per min with differences inside the cylinder of less than ± 3 per cent. β-Rays were filtered out by aluminum.

The cultures were usually exposed to irradiation on the 3rd day after explantation and inoculated with rickettsiae 8 hr and, in some cases, 18 hr after the beginning of irradiation.[3] Since irradiation was carried out at room temperature, control cultures were also maintained at room temperature during the same period.

RESULTS

Microscopic observations of the growth of rickettsiae. Figure 1 is a photomicrograph from a culture inoculated 7 days previously with *R. prowazeki*. The two intensely stained infected cells in the center can easily be distinguished from the surrounding uninfected cells, even at magnifications which do not resolve individual rickettsia. Growth appears to take place primarily in the protoplasmic strands surrounding the fat vacuoles (figure 2) and the general morphology of the infected cell is not greatly altered. This is in contrast to the appearance of similar cells infected with *C. burnetii* which grows in the vacuoles, causes their confluence, and compresses the nucleus (Weiss and Pietryk, 1956). The occurrence of numerous rickettsiae in one plane of focus is common with *R. prowazeki*, but very rare with *C. burnetii*.

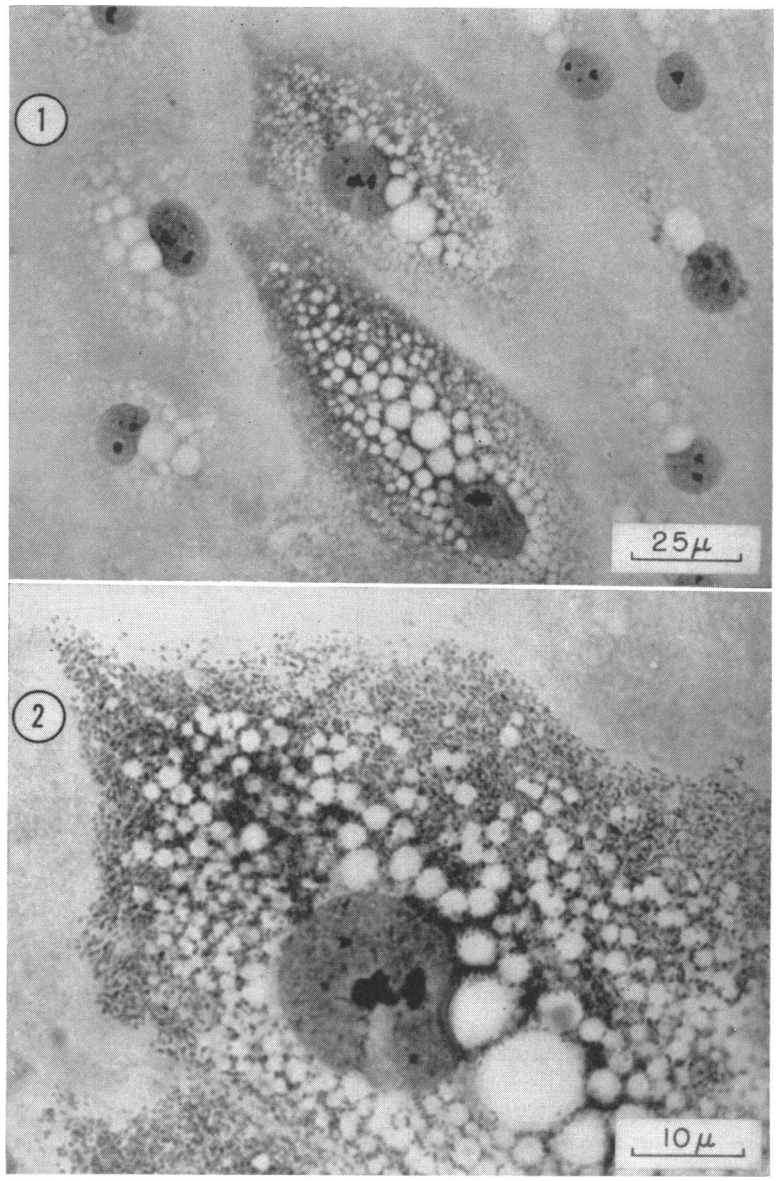
As in the case of *C. burnetii*, infection appeared to spread mainly from cell to cell, rather than uniformly throughout the culture. This spread occurred at variable times during the course of intracellular multiplication and at a variable speed. In spite of the frequent formation of discrete foci of infection, focus counts (Weiss and Pietryk, 1956) performed as for *C. burnetii* did not seem to constitute a practical method of assay for *R. prowazeki*. Great differences were encountered among identically treated cultures, possibly because in many cases the heavily infected cells were released from the glass and light infection in the surrounding cells was easily overlooked.

Infection of the cultures required relatively high concentrations of rickettsiae. In most of the experiments reported below each culture received 10^6 rickettsiae, as measured by chick embryo infectivity, but usually only a few hundred foci of infection were produced.

Effect of Co^{60} γ-irradiation on entodermal cells. Cultures exposed to γ-ray doses of 10,000 to 40,000 r and observed for a period of 7 days did not display any obvious signs of radiation injury. Their microscopic appearance remained essentially the same as that of the unirradiated cells shown in figure 3. This apparent degree of resistance may be attributed to properties of the entodermal cells which possibly overshadowed the effect of radiation. Typical mitosis rarely occurs in these cultures, the nucleolar material is often fragmented even in the unirradiated cells, and the cells are very large and often multinucleated.

With 60,000 r obvious signs of radiation injury appeared and became more marked with increasing doses. The cytoplasm retracted, tearing the epithelial sheet, and some of the cells rounded up and fell from the glass or lost affinity for dyes. Figure 4 is a photomicrograph of a culture which

[3] We are indebted to C. R. Biles, HMC, USN, E. C. Bowman, HM1, USN, and L. A. Freedman, HM2, USN, for irradiating the cultures.



Figures 1 to 5. Photomicrographs from chick embryo entodermal cell cultures stained with May-Gruenwald-Giemsa, taken by P. F. Stables, HMC, USN.

Figure 1. Culture infected with *Rickettsia prowazeki* 7 days previously. Note two intensely stained infected cells at center and lightly stained uninfected cells at either side. 580 ×.

Figure 2. Portion of figure 1 at higher magnification. Note the large number of rickettsiae in the cytoplasm of the entodermal cell. 1500 ×.

had been exposed to 100,000 r 7 days previously. The majority of the cells have already fallen out and the few remaining have spread out in some of the space which has become available. The irradiated cells resemble the unirradiated of an older culture, but do not seem to have other distinguishing morphological characteristics. With doses of 150,000 to 300,000 r the loss of cells from the sheet became more marked and was almost complete with the higher dose. Figure 5 illustrates the densest field found in 3 cultures exposed to 300,000 r 89 hr previously. It contains only a few

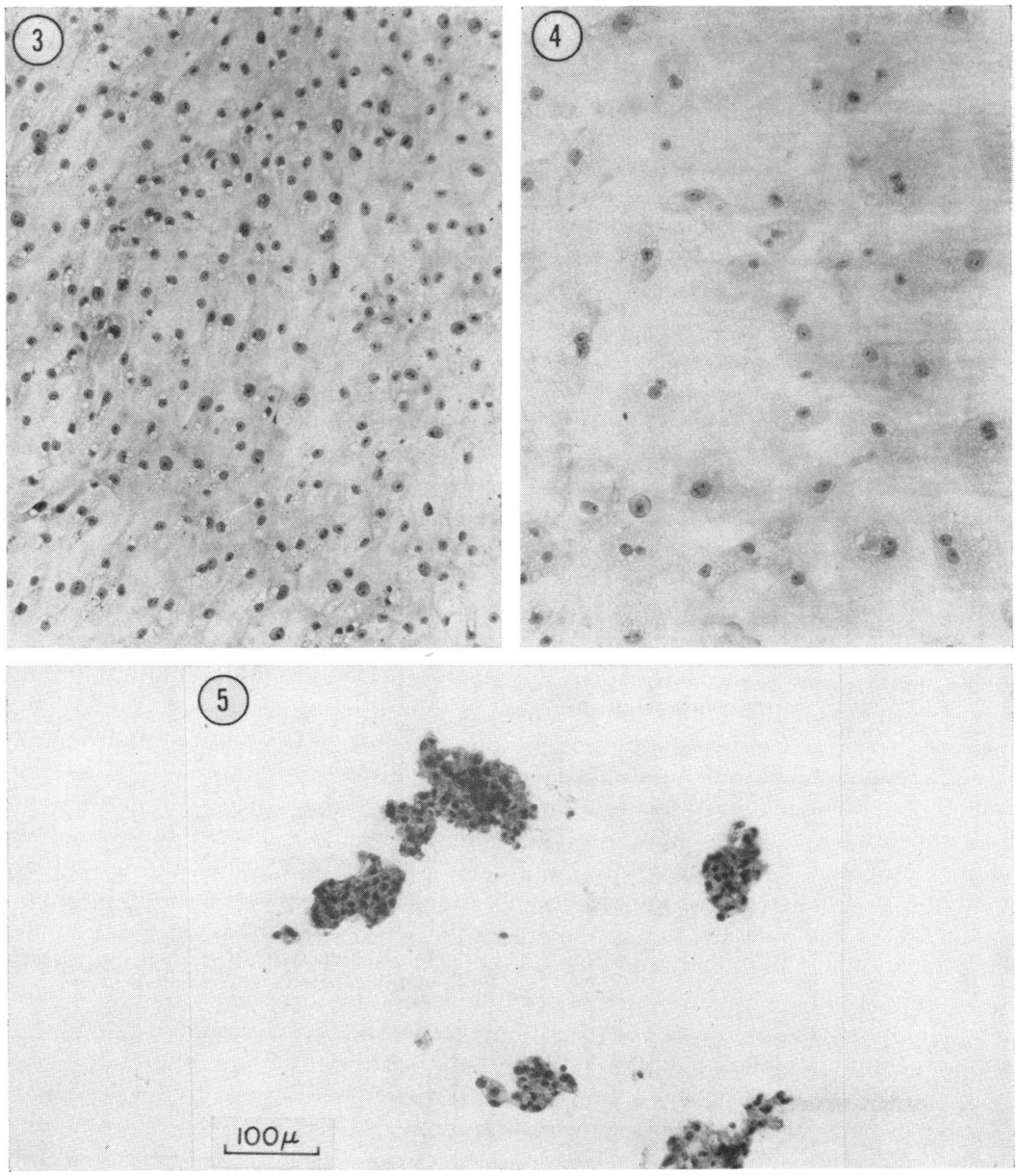


Figure 3. Ten-day culture, unirradiated. 123 ×.
Figure 4. Ten-day culture, exposed to 100,000 r 7 days previously. 123 ×.
Figure 5. Seven-day culture, exposed to 300,000 r 4 days previously. 123 ×.

rounded up cells, which remained after the neighboring cells had disappeared from the glass.

An attempt was made to express quantitatively the effect of radiation by counting nuclei in control and irradiated cultures in 10 fields per culture with an 8 mm objective. The counts were started in fields close to the centers of the cultures and continued towards the peripheries, in order to cover areas of high and low nuclear density in each culture and minimize differences other than those caused by irradiation. The results, shown in table 1, tend to give a conservative estimate of the effect of irradiation, because qualitative changes, such as pyknosis, were not taken into account.

Stability of rickettsiae in tissue culture nutrients.

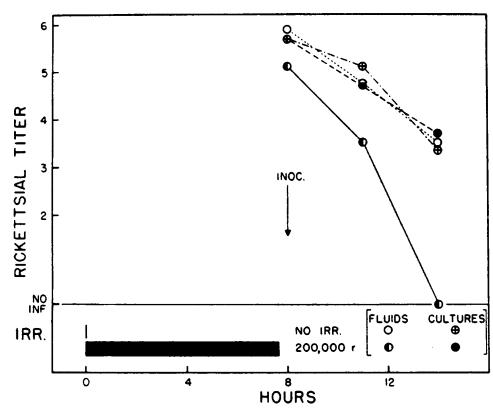
An understanding of the extent of growth of rickettsiae in cell cultures required preliminary determinations of the stability of these microorganisms in the tissue culture nutrients at the 35 C temperature of incubation. One of these experiments is illustrated in figure 6. The activity of rickettsiae, as determined by egg titration, declined rapidly in 3-day culture fluids, which were no longer in contact with the cells. The loss in titer in 6 hr, indicated by open circles, corresponded to approximately 2 logarithms. This rate is considerably greater than that usually obtained in fluids such as the sucrose-phosphate-glutamate solution or skim milk (Bovarnick *et al.*, 1950). The rickettsiae were also suspended in fresh nutrient and added to the 3-day-old cultures. The results, shown by encircled crosses, were almost identical to those obtained with the old nutrient without cells. These results suggest that during the first few hours of cultivation the majority of rickettsiae are inactivated by the heat of incubation and only a few disappear because of absorption into the cells. In some cases, fluids were titrated at 16 and 24 hr after inoculation, but either no or very slight activity could be detected in unirradiated cultures. Therefore, any

TABLE 1

*Percentage nuclei in entodermal cell cultures exposed to varying doses of Co^{60} γ-rays 7 days previously**

Exposure	Nuclei
r	%
0	100
60,000	90
80,000	74
100,000	38
150,000	23
200,000	8
300,000	0

* The procedure for the enumeration of the nuclei is explained in the text.



Figures 6 to 8. Results of infectivity titrations of rickettsiae in chick embryos. The rickettsial titer is expressed as the logarithm of the number of infectious rickettsiae per culture. The time relationship of irradiation to other experimental procedures is indicated by solid bars.

Figure 6. Stability of rickettsiae in tissue cultures and their nutrient fluids. The rickettsiae were suspended in fluids obtained from 3-day cultures, unirradiated (○) and irradiated (◐). The rickettsiae were also suspended in fresh nutrient fluid which was added to the cultures, unirradiated (⊕) and irradiated (●).

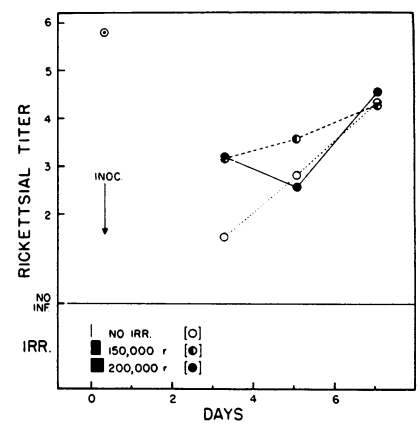


Figure 7. Infectivity titrations of nutrient fluids of unirradiated and irradiated cultures.

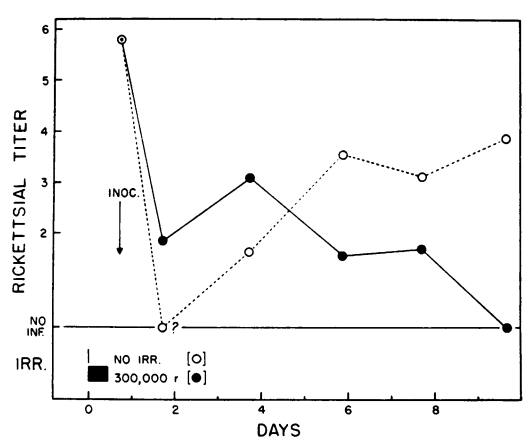


Figure 8. Infectivity titrations of nutrient fluids of unirradiated and irradiated cultures.

activity recovered after the first period of rapid decline can reasonably be considered as rickettsial growth or release of rickettsiae from the cells.

Several experiments, similar to that illustrated in figure 6, were carried out and relatively large variations in the rates of inactivation of rickettsiae were obtained from experiment to experiment. These variations were attributed to differences in pH of the tissue culture fluids. In agreement with Bovarnick *et al.* (1950), it appeared that rickettsiae were more stable at a pH in the neighborhood of 7.0 than at a higher pH.

Rickettsiae suspended in nutrient fluids derived from cultures which had been exposed to 200,000 r ½ hr previously were considerably less stable than in unirradiated culture fluids. As shown in figure 6 and indicated by half-closed circles, no activity was demonstrated after 6 hr, which meant that at least a 10^5 loss in titer had taken place. However, when the rickettsiae were suspended in fresh nutrient and added to the irradiated cultures, figure 6, closed circles, the lower rate of rickettsial decline of the unirradiated groups was established.

The inactivating effect of freshly irradiated tissue culture fluids was further studied. Rickettsiae were suspended in irradiated and unirradiated nutrient fluids which had not been in contact with cells. Differences in rates of inactivation were again noted, suggesting that the cells were not required for the production of the inactivating constituents. However, the inactivating activity elicited by irradiation was soon lost. It could not be demonstrated 10 hr after irradiation in fluids which had or had not been in contact with cultures. Therefore, since the nutrient fluids were replaced in the irradiated cultures at the time of rickettsial inoculation, it can be safely assumed that, under the conditions of our experiments, irradiation did not have any direct effect on the rickettsiae.

Growth of rickettsiae in irradiated and unirradiated cultures. A rapid survey was made of the growth of rickettsiae, for a period of 7 days, in cultures which had been exposed to 10,000 to 100,000 r. Pooled fluids were titrated at 5 and 7 days after inoculation. The results indicated that the fluid of each culture contained approximately 2×10^3 to 3×10^4 infectious rickettsiae. Significant differences between irradiated and unirradiated cultures were not encountered. Microscopic observations were in agreement with the titration experiments.

Figure 7 illustrates the results of an experiment in which 150,000 and 200,000 r were used. The titers of pools of nutrient fluids of irradiated groups obtained at 3 and 5 days were either significantly higher or the same as those of the control cultures, while all the titers were approximately the same at 7 days. These results can best be interpreted by assuming that growth proceeded at the same rate in the 3 groups, but that more rickettsiae were released into the nutrients by the lysing cells of irradiated cultures. At 7 days, the higher release was matched by lower production of rickettsiae by the few surviving cells and the result was a titer similar to that of control cultures. Microscopic observations again supported the results of egg titration. Although the number of cells in the cultures of the various groups varied greatly, the proportions of infected cells were approximately the same and intracellular multiplication was about equally heavy.

The results with 300,000 r, shown in figure 8, were similar. Infectivity could be readily demonstrated at 1 day after inoculation (approximately 6×10^1 infectious rickettsiae per culture) and a moderately high titer (10^3) was obtained at 3 days, at a time when the cells had almost completely disappeared from the glass. The few remaining cells were rounded up and contained pyknotic nuclei, as seen in figure 5. The titers dropped to a low level by the 5th and 7th days after inoculation, but it is remarkable that activity could be demonstrated at all because only 1 to 5 cells per culture could be recognized at these intervals of time. No rickettsial infectivity was detected at 9 days. All the titers of the irradiated groups were significantly different from the corresponding titers of the unirradiated control cultures, higher at 1 and 3 days, lower at 5, 7, and 9 days.

Several experiments were carried out with cultures exposed to 200,000 r, which confirmed and extended the above described findings. Titers higher than those of control cultures were obtained at 2 days and differences became greatest at about the 4th day. By the 8th day after irradiation the titers in the irradiated groups were lower than those of the controls and no activity was demonstrated at 11 days. Unirradiated cultures, on the other hand, continued to release rickettsiae at a slowly diminishing rate for as long as 21 days.

The presence of relatively large numbers of

infectious rickettsiae in the supernatant of irradiated cultures during the first few days after infection can plausibly be attributed to any one of the following three conditions: (a) Early release into the nutrient fluid of rickettsiae grown in the cells on the glass, as in the postulation made above; (b) Growth of rickettsiae in cell debris in the supernatant fluid; and (c) Increased rickettsial stability produced by constituents of host cell disintegration. Experimental evidence indicated that the first condition was the most important one. In one experiment new nutrient and rickettsiae were added to cultures 5 days after irradiation, at a time when they had lost about one half of their cells. Three days later, or 8 days after irradiation, the supernatant fluids contained at least as many infectious rickettsiae as the unirradiated control cultures, but by the 11th postirradiation day, as in a previous experiment, rickettsial activity could not be demonstrated. On the other hand, several attempts to demonstrate either growth or increased rickettsial stability in the supernatant fluids of irradiated cultures failed. It should be noted that the tissue culture fluids of irradiated cultures were generally more alkaline than those of unirradiated cultures and, as expected, were less favorable for rickettsial stability.

DISCUSSION

Weiss and Pietryk (1956) have shown that entodermal cell cultures could readily be infected with *C. burnetii*, provided the inoculum contained at least 300 to 1000 chick embryo ID_{50}. The yield of Q fever rickettsiae from the cultures was not great, 60 times the amount inoculated 7 days previously, but could be increased appreciably by appropriate procedures. The results with *R. prowazeki* were similar to those obtained with *C. burnetii*, except that the yield of typhus rickettsiae from these cultures, even under the best conditions, was lower than from the inoculum. These results can be attributed, in part, to the greater instability of extracellular typhus rickettsiae in tissue culture fluids. However, it is possible that the growth of *R. prowazeki* may be enhanced by relatively simple modifications of the cultural procedure. For example, Blackford and Weiss (1958, *in preparation*) have shown that the addition of moderate amounts of sodium pyruvate, 1.25 to 2.5 mmoles, to the medium enhanced the growth of *C. burnetii*.

The high degree of resistance of the entodermal cells to ionizing radiations was somewhat surprising. Although a direct comparison with the work of other investigators cannot be made, it can be said that entodermal cells are far more resistant to the effect of Co^{60} γ-rays than HeLa cells to X-rays (Syverton, J. T., *personal communication*). However, Morgan Harris (*personal communication*) pointed out that avian cells are appreciably more resistant to ionizing radiations than mammalian cells.

The results of experiments with infected irradiated cells suggest that irradiation did not enhance rickettsial growth, despite the fact that the yield of rickettsiae was greater in the irradiated than in the control cultures during the first few days of incubation. The view that this greater yield was due to lysis of the host cells and earlier release of the microorganisms was supported by the fact that it coincided with the destruction of the cells and was not demonstrated under other conditions. It was also supported by experiments, not reported elsewhere, in which the cells were disrupted by other means. In two experiments the nutrient fluids were replaced by the sucrose-phosphate-glutamate solution containing 1 per cent bovine plasma albumin, at the time of rickettsial inoculation in one case, on the 5th postinoculation day in the other. The cells were destroyed over a period of 2 or 3 days, in a manner somewhat similar to the destruction which followed irradiation. Rickettsial titers during this period were the same or higher than those of the cultures which had been maintained on the usual nutrient.

The conditions of these experiments were so different from those of other investigators (Zinsser and Castaneda, 1932; Liu *et al.*, 1941; Kawamura and Shimizu, 1955; and Greiff *et al.*, 1953, 1955, 1957*a*, *b*), who demonstrated that X-irradiation enhanced the growth of rickettsiae in experimental animals and chick embryos, that a comparison is not possible. However, the possibility should not be discounted that in experimental animals, as well as in tissue culture, rickettsiae are released more rapidly from cells injured by irradiation than from unirradiated cells, and that this release might be a factor favoring invasion of new cells in irradiated animals.

The main question arising from this work is the extent to which a cell can be injured by ir-

radiation and still be able to support the growth of typhus rickettsiae. It is quite obvious, from the results obtained, that the degree of cellular injury can be very high and that host cells may possibly continue to support the growth of rickettsiae as long as they maintain any semblance of organized structure. In this connection it appeared desirable to determine whether other intracellular microorganisms would grow as well in irradiated cells. A similar series of investigations, with exposures up to 100,000 r, were carried out with the 6BC strain of psittacosis virus. Growth of the virus was determined by microscopic observations of stained cultures fixed over a period of 10 days after inoculation. Psittacosis virus was apparently able to grow in irradiated as well as in unirradiated cells. Therefore, it appeared unlikely that the properties of the two microorganisms could be differentiated by their ability to grow in cells injured by irradiation.

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SUMMARY

The growth of *Rickettsia prowazeki* (Madrid E strain) in unirradiated and irradiated cultures of chick embryo entodermal cells was investigated. Monolayer entodermal cell cultures were prepared on glass without plasma from explants of avascular membranes of 4-day-old chick embryos. Three days after explantation the cultures were exposed to 10,000 to 300,000 r of Co^{60} γ-radiations. The smallest dose which produced widespread morphological changes was 60,000 r. The cells disappeared from the glass or lost affinity for dyes at higher exposures in the following percentages: 60 per cent with 100,000 r in 7 days, 90 to 95 per cent with 200,000 r in 7 days, 99 to 100 per cent with 300,000 r in 4 days. The nutrients of irradiated and control cultures were replaced, 8 to 18 hr after the beginning of irradiation, with nutrients containing high concentrations of partially purified rickettsiae. The growth of the microorganisms was followed for 7 days by examination of stained cultures and titration of fluids in eggs. Infection of the cultures required relatively high concentrations of rickettsiae, but a moderate amount of growth was obtained in unirradiated cultures. Cell cultures that had received 10,000 to 100,000 r supported the growth of rickettsiae to approximately the same extent as unirradiated controls. With doses of 150,000, 200,000, and 300,000 r greater numbers of rickettsiae were released into the nutrients, during the first few days of infection, than in control cultures. Rickettsial titers became lower than those of the controls, however, when almost all the entodermal cells had disappeared from the glass. The results suggest that irradiation did not enhance rickettsial multiplication, but simply elicited a more rapid release of the microorganisms during the period in which the host cells lysed.

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