perimental salmon. Two control dogs were fed noninfected kidneys at 12, 24, and 33½ months. These dogs remained asymptomatic for at least 60 days. The same dogs were later proved susceptible to salmon disease when fed infected fish from the Elokomin River.

The persistence of a sub-microscopic disease agent in an endoparasite has been studied by Shope (*7*) who reported that swine influenza virus persisted for at least 2 years in the immature lungworms of swine.

Recently, we recovered a Chinook salmon (*Oncorhynchus tshawytscha*) returning to the Elokomin River of Washington with "sea lice" still attached. (These lice drop off soon after the salmon return to fresh water). This fish was marked at the Elokomin River Hatchery and was 5 years of age; presumably, it was returning after having spent 4 years in the ocean. Viable metacercariae were seen in the kidneys of this fish on microscopic examination. The kidneys of the fish were fed to a susceptible dog which became sick and died of typical salmon disease. Adult flukes capable of transmitting rickettsiae were demonstrated on postmortem examination. It was unlikely that this fish was reinfected on its return migration because the presence of "sea lice" indicates recent return from salt water and it is known that the cercariae have to remain in the fish tissue for approximately 10 days before they become infective for the dog.

We conclude that the metacercariae of *Nanophyetus salmincola* containing *Neorickettsiae helminthoeca* can persist for at least 33½ months in fish in sea water.

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Tetracycline Fluorescence in Permeability Studies of Membranes around Intracellular Parasites

Abstract. *Certain protozoa, bacteria, and viruses when phagocytosed by host cells become surrounded by an intracytoplasmic boundary. This membrane prevents the fluorescent antibiotic tetracycline from entering the parasites when it is added to the medium, since they show no fluorescence, whereas extracellular parasites are immediately visible. As soon as the host cell dies, the intracellular parasites also become visible. This indicates that the boundary probably is of host origin. This phenomenon provides a means for selective permeability studies of such boundaries. A similar exclusion of tetracycline from certain extracellular parasites is seen in the presence of whole serum.*

It was reported previously (*1*) that tetracycline is selectively accumulated by mitochondria of living cells in tissue culture and in vivo, but that nuclei, vacuoles, interparticulate cytoplasm, and cell boundaries appear dark when the tetracycline distribution is observed by fluorescence microscopy. This indicates that of the four boundaries involved, two are very permeable (cell and mitochondrial membrane) and two are slightly permeable (nuclear and vacuolar membrane) to the drug.

For the study of intracellular parasites reported herein, host cells were allowed to attach to coverslips in small petri dishes containing the appropriate medium. After the cells adhered, a suspension of parasites was added so that phagocytosis could take place. Five minutes before observation a tetracycline solution, adjusted to *p*H 7 (final concentration, 50 to 200 μg/ml), was added. The coverslip was then applied to a tissue culture chamber or placed on a slide and observed under a microscope (*2*). Photomicrographs were made of the same preparation; first by phase contrast to localize the parasites, then by fluorescence microscopy to determine whether the parasites fluoresced. In each case it was shown that the extracellular parasites or the internal parasites in moribund host cells did fluoresce.

The results obtained in typical preparations are illustrated in Figs. 1 to 5. Inclusion *Blennorrhea* (*3*) was used as a representative large, complex virus, *Salmonella typhosa* (strain Ty 2) as a bacterium capable of intracellular multiplication, *Escherichia coli*, strain S, and *Bacillus cereus* (recently isolated) as representatives of Gram-negative and Gram-positive bacteria, which could be stained with tetracycline and were capable of being phagocytosed, and *Toxoplasma gondii* (*3*) as an intracellular protozoan.

Figure 1, *A* and *B*, show human synovial cells, infected with inclusion *Blennorrhea* virus suspended in Eagle's

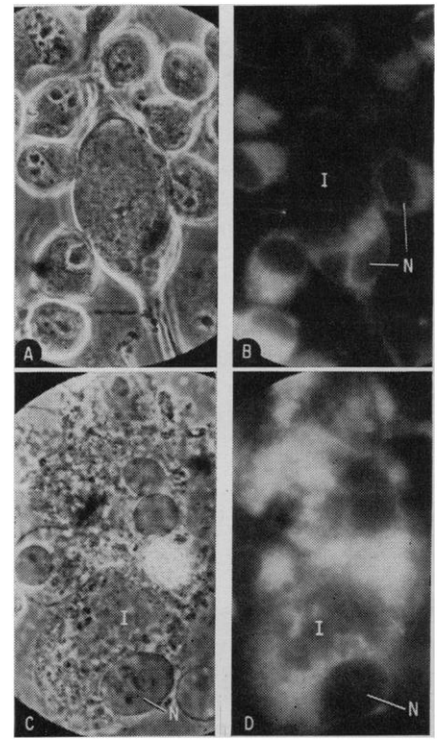

Fig. 1. Photomicrographs of *Blennorrhea* in living human synovial cells, shown (*A*) by phase contrast, and (*B*) after staining with fluorescent tetracycline; (*C*) and (*D*) show the same, but moribund cells. *I*, Intracellular inclusion body; *N*, nucleus.

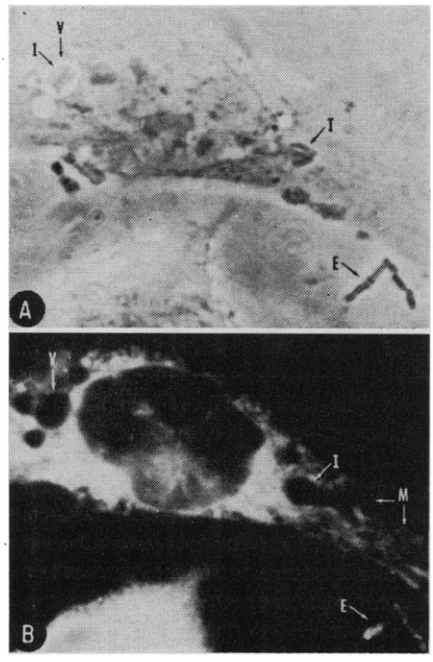

Fig. 2. *Salmonella typhosa* in living mouse L cells, shown (*A*) by phase contrast, and (*B*) by tetracycline fluorescence. *V*, Vacuole with *I*, intracellular bacteria; *E*, extracellular bacteria; *M*, mitochondria.

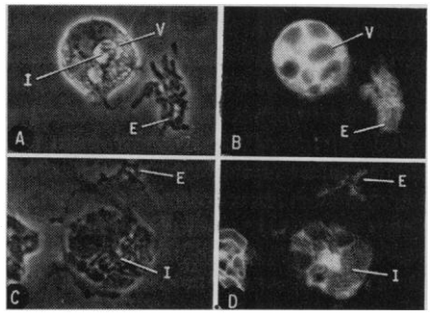

Fig. 3. *Escherichia coli* in living guinea pig phagocytes, shown (*A*) by phase contrast, and (*B*) by tetracycline fluorescence; (*C*) and (*D*) show the same, but moribund cells; *I*, *E*, and *V*, as in Fig. 2.

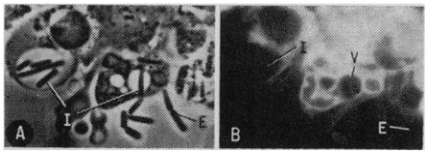

Fig. 4. *Bacillus cereus* in living and moribund guinea pig phagocytes, shown (*A*) by phase contrast, and (*B*) by tetracycline fluorescence; *V*, vacuole with bacterium in living cell, *I*, vacuole with bacteria in moribund cell, *E*, extracellular bacteria.

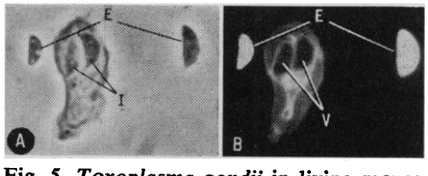

Fig. 5. *Toxoplasma gondii* in living mouse phagocytes, shown (*A*) by phase contrast, and (*B*) by tetracycline fluorescence; *I*, *E*, and *V*, as in Fig. 2.

medium No. 2, with 10 percent horse serum and 5 percent glucose (*3*). The typical virus inclusion body (*I*) showed vigorous Brownian movement. In Fig. 1*B*, neither the nuclei (*N*) nor the inclusion body (*I*) showed fluorescence, whereas the round mitochondria of the host cell showed clear yellow fluorescence. In Fig. 1, *C* and *D*, a similar culture is shown, moribund as the result of being in contact with distilled water for 5 minutes. The cells were swollen and the nuclei rounded, and an inclusion body (*I*) with decreased Brownian movement now showed clear fluorescence. This suggested that although the inclusion body contained material which could be stained with tetracycline, it was not stained while surrounded by a healthy host membrane. Litwin (*4*) states that he could not observe a membrane surrounding trachoma virus particles 30 hours after infection. However, such a membrane may be demonstrable by the use of tetracycline.

In Fig. 2, mouse L cells are shown in Eagle's medium with 5 percent horse serum 30 minutes after they were exposed to a suspension of *S. typhosa*. Fluorescence microscopy indicates the presence of a tetracycline-excluding boundary about all intracellular bacteria. By contrast, the extracellular bacteria are fluorescent. A closely adherent boundary, observed occasionally, may explain reports that bacteria are sometimes seen free in the cytoplasm. Electron microscope studies have also shown the presence of boundaries around intracellular bacteria in fixed preparations (*5*). The assertion that these boundaries represent the host cell boundary (plasma membrane) (*6*) does not correspond with our observations (*1*) that the latter is permeable to tetracycline in either direction. However, Goodman and Moore (*6*) mention that subsequent to engulfment of the parasites considerable changes seem to occur in this membrane.

In Fig. 3, guinea pig phagocytes from peritoneal washings were suspended in Eagle's medium and exposed to a suspension of *E. coli* for 30 to 60 minutes at 37°C. By phase microscopy (Fig. 3*A*) the intracellular bacteria (*I*), in a vacuole (*V*), as well as extracellular bacteria (*E*), are visible. Under fluorescence microscopy (Fig. 3*B*) only the extracellular bacteria are visible. However, in swollen, dying cells in which vacuoles are forming (Fig. 3, *C* and *D*) both groups are visible. Evidently, a barrier operating only in the living host cell prevented the uptake of tetracycline by the intracellular bacteria. In Fig. 4, *A* and *B*, the same phenomena are shown with *B. cereus*.

In Fig. 5, an intracellular and extracellular protozoan *Toxoplasma gondii*, maintained in mice, is shown in mouse phagocytes, suspended in phosphate-buffered saline. Under fluorescence microscopy only two vacuoles (*V*) can be seen at the locus of the intracellular parasites. The boundary is so closely adherent to the parasite that it cannot be recognized under the phase microscope. Under the fluorescence microscope this boundary is visible, presumably due to the presence of mitochondrial elements of the host.

When the exposure to tetracycline was prolonged, some of the parasites were phagocytosed after they had taken up tetracycline. They were then visible under the fluorescence microscope. This, as well as the mitochondrial fluorescence, indicated that the intracellular localization of the parasites does not cause the fluorescence of tetracycline to be obscured because of absorption of the activating radiation by the surrounding medium.

A further study on the nature of boundaries similar to those described above was possible when it was observed that *Toxoplasma* in 100 percent mouse serum, in the presence of 100 μg of tetracycline per milliliter was invisible, as was this parasite in the intracellular environment. Similarly, *Trypanosoma lewisi* was invisible in rat serum, but fluorescent when kept in saline. The effects of variation in the external medium, including the presence of specific antibodies, on the degree of fluorescence have been studied in more detail with *T. lewisi*, and have been reported briefly elsewhere (*7*).

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Toxic Effect of Pseudomonas tabaci on RNA Metabolism in Tobacco and Its Counteraction by Kinetin

Abstract. *Treatment of intact tobacco leaves with toxin-containing culture filtrates of* Pseudomonas tabaci *markedly reduced the ribonucleic acid content of the leaves. The decrease was counteracted by spraying the leaves with 10^{-4}M kinetin. Simultaneously with the decrease in RNA, the ribonuclease activity of extracts from tissues treated with toxin increased. Experiments on the effect of kinetin on the ribonuclease activity of toxin-treated tissues gave no consistent results.*

The toxin of *Pseudomonas tabaci* is a structural analogue of methionine (*1*). It has been shown by Braun in model experiments with *Chlorella vulgaris* that, as expected, the effect of the toxin can be counteracted by methionine. However, methionine failed to antagonize the toxin in tobacco (*2*). Therefore, further studies on the mode of action of the wildfire toxin seemed necessary. To this end, we investigated the effect of toxin-containing filtrates of *P. tabaci* cultures on the metabolism of the host. It has been found (*3*) that the amount of soluble proteins decreases upon treatment of the host tissues with the toxin.

Table 1. RNA content of tobacco half-leaves treated with toxin-containing culture filtrate of *Pseudomonas tabaci* and with nutrient medium, respectively.

Expt. No.	RNA content (mg/g fresh wt.)	
	Treated with medium	Treated with filtrate
1	1.54	0.83
2	1.20	0.69
3	1.35	0.95

Table 2. Effect of kinetin on the RNA content of tobacco half-leaves treated with toxin-containing culture filtrate of *Pseudomonas tabaci.*

Expt. No.	RNA content (mg/g fresh wt.)	
	Effect of water	Effect of $10^{-4}M$ kinetin
1	1.00	1.54
2	1.16	1.60
3	0.76	0.95

The protein concentration could be maintained at the level of the control by spraying toxin-treated leaves with kinetin (*3*). In view of the well known effect of kinetin on RNA metabolism (*4–6*) and because of the close connection between protein and RNA metabolism, we investigated the influence of toxin-containing culture filtrates on the RNA content of host tissues.

Intact half-leaves of *Nicotiana tabacum* "White Burley" plants were injected with toxin-containing filtrates of *P. tabaci* cultures. Control half-leaves were injected with Czapek's medium. After an incubation period of 4 to 5 days under ordinary greenhouse conditions disks were punched from the leaves for spectrophotometric determination of RNA content and ribonuclease activity. In the determination of RNA the procedure described by Osborne (*5*) was followed. The method adopted for the determination of ribonuclease activity was based on the principles described by McDonald (*7*) and Tuve and Anfinsen (*8*). Tissue extracts corresponding to 10 mg of fresh weight per milliliter were incubated in the presence of 0.1*M* acetate buffer, *p*H 5.0, with yeast RNA in a final concentration of 0.1 percent at 37°C for 30 minutes. The reaction was stopped by McFadyen's reagent, the reaction mixture was centrifuged, and after five-fold dilution of the supernatant the optical density was determined at 260 mμ. One enzyme unit corresponded to an increase in optical density of 0.010 over the zero time control (*8*).

Treatment of tobacco leaves with culture filtrates of the pathogen grown for 48 hours in Czapek's medium resulted in a decrease in RNA content (Table 1). This clearly indicates that the toxin damages the RNA metabolism of the host.

Kinetin counteracts the breakdown of RNA in detached leaves (*5*). Therefore, experiments were conducted to find out whether or not the toxin-induced damage to RNA metabolism can also be counteracted by kinetin. Intact whole leaves were injected with culture filtrates of *P. tabaci.* Half-leaves were sprayed three times in 24-hour intervals with $10^{-4}M$ kinetin; controls were sprayed with water. The toxin-induced decrease of RNA was inhibited in the kinetin-treated half-leaves (Table 2).

The regulation of the RNA content of plants is poorly understood. Although no conclusive evidence has been presented, it has been suggested repeatedly that ribonuclease may participate in the regulatory process. Therefore, the ribonuclease activity of extracts from half-leaves treated with toxin and from control half-leaves was compared. Ribonuclease activity was increased by about 100 percent in the tissues treated with culture filtrate (Table 3). Attempts to counteract by kinetin the toxin-induced increase in ribonuclease activity gave no consistent results (Table 4), in contrast to the highly reproducible effect of kinetin on the concentration of RNA. Thus, there is no clear-cut correlation between the ribonuclease activity of the tissue extracts and their RNA content.

Table 3. Ribonuclease activity in tobacco half-leaves treated with toxin-containing culture filtrate of *Pseudomonas tabaci* and with nutrient medium, respectively.

Expt. No.	Ribonuclease activity (enzyme units)	
	Treated with medium	Treated with filtrate
1	86	156
2	69	132
3	45	66

Table 4. Effect of kinetin on the ribonuclease activity of tobacco half-leaves treated with toxin-containing culture filtrate of *Pseudomonas tabaci.*

Expt. No.	Ribonuclease activity (enzyme units)	
	Effect of water	Effect of $10^{-4}M$ kinetin
1	63	50
2	33	29
3	52	55

The evidence presented above indicates that an important aspect of the mode of action of the wildfire toxin is damage to the RNA metabolism of the host. The effect is not based on the parasitically induced increase in ribonuclease activity.

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