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The development of TRIC organisms in cell cultures during multiple infection

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SUMMARY

The relationship between multiplicity of infection and the yield of organisms and of polysaccharide was studied in BHK 21 cells infected with TRIC organisms. Although an increase in the multiplicity of infection resulted in an increase in the absolute number of organisms per culture, there was a decrease in relation to the number of infecting organisms. The amount of polysaccharide produced was independent of multiplicity of infection; it was not limited by the concentration of glucose in the medium. Polysaccharide leaked from cells and was slowly broken down in the culture medium.

INTRODUCTION

Although the agents of trachoma and inclusion conjunctivitis are usually isolated and grown in the chick embryo yolk sac, many workers have employed cell cultures (e.g. Bernkopf, Treu & Maythar, 1964; Gordon, Dressler & Quan, 1967; Jenkin, 1966). Cell cultures offer many advantages over the chick embryo yolk sac; infective organisms can be titrated with greater precision and they yield more easily purified material for biochemical investigations.

Within the genus *Chlamydia* an important species characteristic is the presence or absence in the inclusion of a carbohydrate which is probably glycogen (Gordon & Quan, 1965). The role of this material in the multiplication of the organism has not been elucidated, nor is it certain whether it is produced by the organism or the host cell (see Moulder, 1964).

Recently Blyth & Taverne (1972) observed that inclusions in multiply infected cells coalesce, and at a given time appear larger and more advanced than those derived from infection by a single organism. It seemed possible that in multiply infected cells developmental processes act synergistically. To improve our understanding of such processes, a quantitative study was made of the effect of multiplicity of infection on the number of elementary bodies produced, their infectivity and the yield of carbohydrate. This information is also of practical importance when large amounts of material are required, for example in biochemical studies or for production of vaccines.

MATERIALS AND METHODS

Strain

TRIC/ /China/Peking – 2/OT*f* (T'ang, Chang, Huang & Wang, 1957) was used; the strain is designated according to the system proposed by Gear, Gordon, Jones & Bell (1963) and the suffix *f* indicates a 'fast-killing' variant (Reeve & Taverne, 1964).

Preparation of pools

Suspensions of infected yolk sacs were treated with KCl and stored at −70° C. (Taverne & Blyth, 1971).

Cell cultures

Monolayers of BHK 21 cells (Stoker & Macpherson, 1964) were seeded into Leighton tubes, 30 ml. or 250 ml. plastic bottles (Falcon Plastics) the day before infection. Growth medium was Eagle's tissue culture medium (BHK) (Wellcome Reagents Ltd) containing 0·035 % $NaHCO_3$, 10 % tryptose phosphate broth (Difco Laboratories) and 10 % calf serum (Flow Laboratories Ltd). The maintenance medium (MM) used after infection was growth medium without serum and contained streptomycin (100 μg./ml.). It was buffered to pH 7·5 with tris and HCl to a final concentration of 0·05 M tris.

Reagents

Phosphate-buffered saline (PBS) (Dulbecco & Vogt, 1954) without calcium and magnesium was used for washing cell monolayers.

Disodium ethylenediamine tetracetate (E.D.T.A.) 0·002 % in PBS was used to remove cells from the surface of bottles. Borate buffer pH 7·0 consisting of 0·02 M-H_3BO_3, 0·0008 M-$Na_2B_4O_7$, 0·0007 M-$CaCl_2$, 0·003 M-$MgCl_2$ and 0·15 M-NaCl in distilled water was used to resuspend cells after they were stripped from flasks.

Anthrone reagent was prepared by dissolving 0·5 g. anthrone (Hopkin and Williams Ltd) in a mixture of 500 ml. concentrated sulphuric acid (Analar) and 145 ml. distilled water (Mokrasch, 1954). It was stored at 4° C.

Assay of polysaccharide

Samples of infected and uninfected material were boiled with an equal volume of 10 N-KOH for 1 hr. to destroy any free glucose and then neutralized by glacial acetic acid (van der Vies, 1954). After suitable dilution, 1 ml. samples were heated with 6 ml. anthrone reagent at 80° C. for 40 min. and their optical density at 620 nm. was measured. The material measured in this procedure is referred to throughout as polysaccharide. Standard curves were made for each experiment with glucose (Analar B.D.H.) and oyster glycogen (B.D.H.). As little as 5 μg./ml. glucose could be detected with this assay method. Results are expressed in terms of glucose equivalents.

Infection of cell cultures and titration of infective organisms

Monolayers of BHK cells were infected with TRIC organisms in MM by centrifugation at 600 ***g*** for 30 min. at 35° C.; the medium was then replaced with new MM and the cultures were incubated at 35° C.

Inclusions were counted in cultures fixed with methyl alcohol and stained with Giemsa; the results were expressed as inclusion-forming units (IFU)/ml.

Particles were counted by a darkground method (Reeve & Taverne, 1962).

Design of experiments

Some uninfected BHK cultures were fixed and stained at the time of infection to determine the number of cells present. Other cultures were infected with various concentrations of TRIC organisms. The inoculum was also titrated so that the exact multiplicity of infection could be calculated retrospectively. After incubation for various periods some cultures were fixed to count the inclusions; in others the content of total particles, infective organisms and polysaccharide was assayed by the following methods.

Yield of new organisms. Culture vessels were incubated at 35° C. for 5 min. with E.D.T.A. and were then examined microscopically to ensure that all the cells had been removed from the surface. The cells were disrupted ultrasonically at 4° C. in a Soniprobe tank type 1130/2A (Dawes Instruments Ltd) at stage 8 for 30 sec. The disrupted material was then assayed for infectivity and for total particles.

Yield of polysaccharide. The MM from monolayers in 250 ml. flasks was discarded and the cells were stripped from the flasks into ice-cold borate buffer by rolling glass beads over the surface. The cell suspension was then disrupted with a mechanical shaker (Baird and Tatlock Ltd) at full speed for 2 min. If the polysaccharide was not assayed immediately the homogenized cell suspension was stored at −40° C.

RESULTS

Forty-five hours after inoculation inclusions resulting from infection with one organism differed strikingly in appearance from those derived from multiple infections (Plate 1).

Cultures that had been infected with < 1, 1·6, 8 or 16 organisms per cell were disrupted 28, 36, 42 and 48 hr. after infection and the numbers of infective organisms were titrated.

The yield of infective organisms from each inclusion increased with multiplicity of infection (Fig. 1). After 42 hr. inclusions resulting from infection with one organism contained on average 278 IFU whereas those receiving 16 infective organisms contained 848 IFU. Inoculation of more than 100 organisms per cell caused a cytopathic effect.

By contrast, increases in multiplicity of infection diminished the yield in relation to the number of infective organisms inoculated (Fig. 2). For instance, 42 hr. after infection, when all yields were attaining their maxima, the ratio of the yield

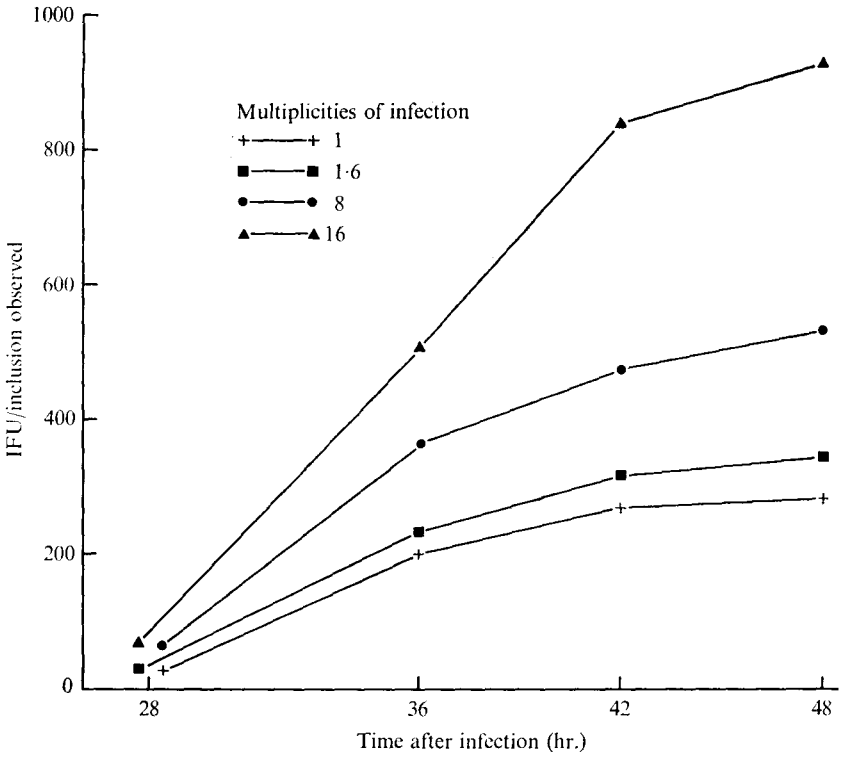

Fig. 1. The yield of infective organisms expressed as IFU/inclusion observed at each time.

Table 1. *Effect of multiplicity of infection on the yield of infective organisms and total particles per cell 42 hr. after infection*

Multiplicity of infection	IFU*	Mean	Total particles*	Mean
< 1	289 267	278	331 303	317
1·6	329 311	320	378 324	351
8	493 465	479	435 413	424
16	895 801	848	973 867	920

* Mean of two replicates.

of infective organisms to the number of infective organisms in the inoculum was 280 to 1 when the multiplicity of infection was 1 and 60 to 1 when the multiplicity was 16.

At all times, within the limits of experimental error, every new particle formed was infective. Table 1 gives the figures for 42 hr.

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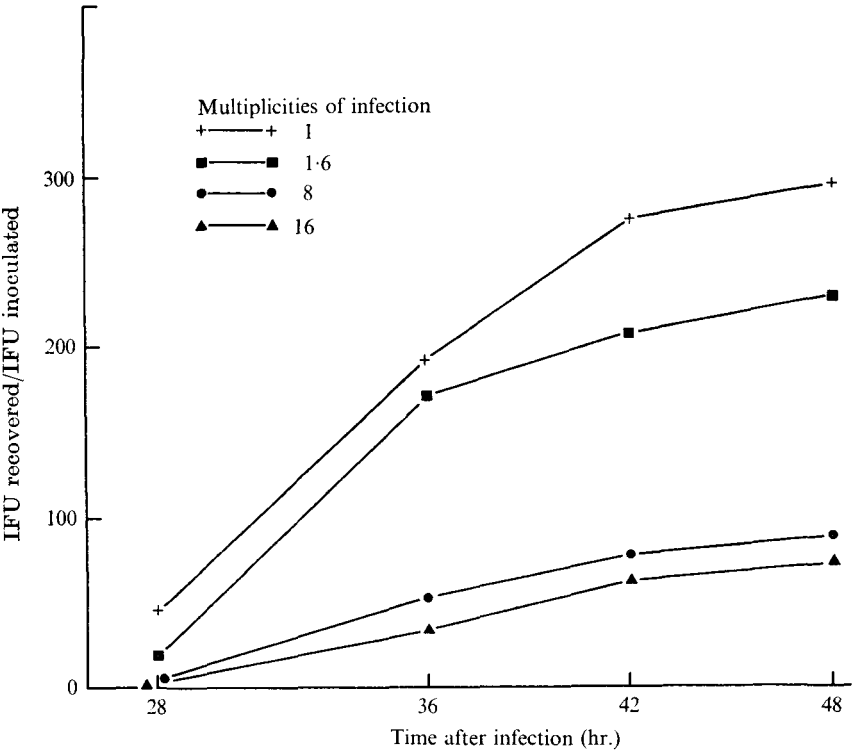

Fig. 2. The yield of infective organisms expressed as IFU recovered/IFU inoculated.

Yield of polysaccharide

Experiments were done to determine whether the amount of polysaccharide produced in infected cells varied with the multiplicity of infection in the same way as the number of organisms.

Polysaccharide was measured 29, 40, 48 and 56 hr. after infection of cultures with the same numbers of organisms per cell as before (Fig. 3). In uninfected cells the amount of polysaccharide did not vary with time. At no time did the amount per infected cell vary with multiplicity of infection. Polysaccharide was first detected 28 hr. after infection and the amount increased thereafter to reach a maximum, at 48 hr., of 115 μg. glucose equivalents per 10^6 infected cells. By 56 hr. the amount in the cells had decreased to 64·3 μg. glucose equivalents per 10^6 infected cells.

Effect of glucose concentration on the production of polysaccharide

The amount of polysaccharide formed by an infected cell at any time may have been constant, whatever the multiplicity of infection, because it was limited by the amount of glucose in the culture medium. The concentration of glucose in the medium of cultures infected with 8 organisms per cell was increased from 4·5 g./l. to 9 g./l., either immediately after inoculation, or 28 hr. later. The amount of polysaccharide formed was measured 42 hr. after infection.

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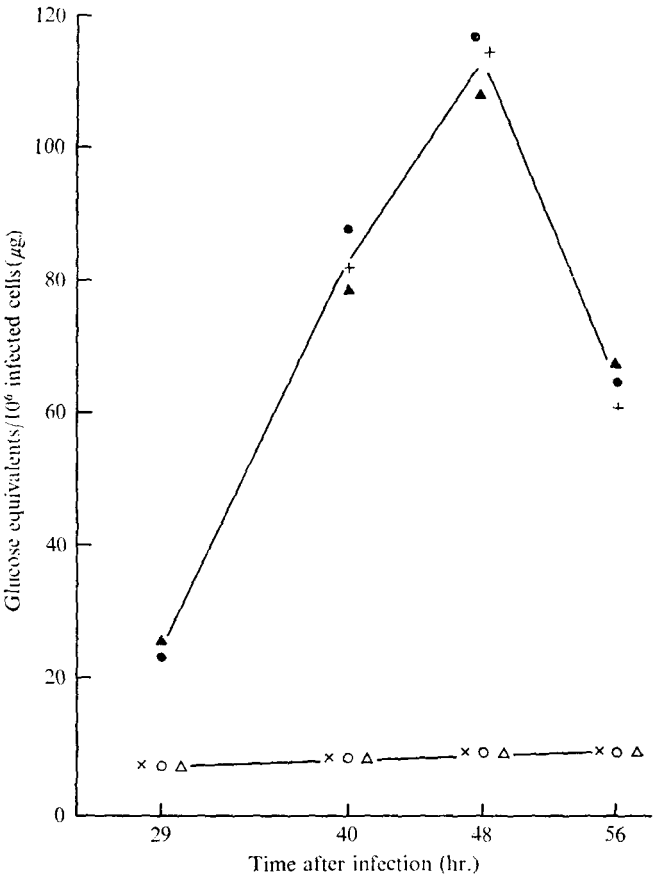

Fig. 3. Polysaccharide in infected and uninfected cultures. Key as in Fig. 1; closed and open symbols represent polysaccharide in infected and control uninfected cultures respectively.

The addition of more glucose to the medium did not affect the amount of polysaccharide formed per infected cell: there were 87·2 μg. glucose equivalents per 10^6 infected cells in the control cultures; 84·2 μg. when MM containing increased glucose was added immediately after infection and 88·2 μg. when it was added 28 hr. later.

Loss of polysaccharide from infected cells

The amount of polysaccharide in infected cells began to decrease 48 hr. after infection. It may have been degraded within the cell or may have leaked out of them. To test the second possibility the amount in the medium was measured at various times after infection.

Cultures were inoculated with eight organisms per cell and 30, 48 and 56 hr. after infection the MM was removed from these and similar control cultures and replaced with 5 ml. of fresh MM, the smallest volume that covered the monolayer. After incubation at 35° C. for 90 min. the polysaccharide in the new medium was assayed.

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The amount and proportion of polysaccharide in the medium of uninfected cultures remained fairly constant. The amount in the medium of infected cultures was higher, but varied little with time; the proportion in the medium was least 48 hr. after infection, when the amount in the cells was greatest (Table 2).

Stability of polysaccharide in MM

The possibility that degradation of free polysaccharide in MM at 35° C. resulted in an underestimate of the true amount released by the infected cells was tested. A lysate was prepared from cells infected 48 hr. earlier and was heated at 100° C. for 20 min. to destroy enzymic activity; this treatment did not alter the amount of polysaccharide present.

Samples were then incubated at 35° C. for 6 hr. in the media listed (Table 3). Polysaccharide was assayed before and after incubation and a comparison was made with the stability of oyster glycogen in medium from infected cultures.

The amount of polysaccharide remained the same after incubation in either new or heated MM; in unheated media from infected and from uninfected cells the losses were respectively 32 % and 15 %. When oyster glycogen was incubated in medium from infected cultures a 42 % loss was observed. The values obtained for the amount of polysaccharide in culture medium after 90 min. incubation are thus probably underestimated by the order of 5 %, assuming that the rate of degradation is constant.

Table 2. *Polysaccharide in culture medium of infected and uninfected cultures*

	Uninfected μg. glucose equivalents/10^6 cells		Infected (8 IFU/cell) μg. glucose equivalents/10^6 cells	
Time	Monolayer	Medium	Monolayer	Medium
30	7·3	2·5	23·8	10·5
48	8·8	2·1	115·0	11·5
56	9·1	2·6	64·3	15·1

Table 3. *Stability of polysaccharide during 6 hr. incubation in MM*

Source of polysaccharide	Source of MM	Heated†	μg./ml. glucose equivalents of polysaccharide 0 hr.	6 hr.	% remaining at 6 hr.
Infected cell lysate*	New	No	82·5	85	103
	Infected culture	Yes	85	85	100
	Infected culture	No	82·5	56·2	68
	Uninfected culture	Yes	87·5	90	103
	Uninfected culture	No	85	72·5	85
Oyster glycogen	Infected culture	Yes	150	153	102
	Infected culture	No	150	87·5	58

* Cells infected 48 hr. earlier were disintegrated by shaking with glass beads; the lysate was heated at 100° C. for 20 min.

† 100° C. for 20 min.

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DISCUSSION

Although many workers have studied the growth cycle of TRIC organisms only Furness & Fraser (1962) measured the yield of infective organisms from an inclusion. Using HeLa cells in conditions designed to ensure infection with only one organism, they obtained a maximum of 35–60 infective organisms per inclusion 34–38 hr. after infection. This figure is lower than the maximum of 278 reported here for BHK cells, but experience in this laboratory suggests that the difference is probably due to improvements in the method of titration.

These findings show that, within limits, increasing the multiplicity of infection resulted in an increase in the absolute number of organisms within each infected cell, and that these organisms were all infective on each occasion that they were tested, starting 28 hr. after inoculation. This finding is of practical value in devising conditions to give the greatest yield of infective organisms from cell cultures.

The observation that with multiple infection the yield of organisms is diminished in relation to the number of organisms inoculated, indicates that some kind of interference or competition occurs within the multiply infected cell.

The nature and function of the polysaccharide present in inclusions is not known. It stains brown with iodine and is frequently referred to as glycogen, but there is no evidence that glucose is the only sugar present or that the polysaccharide is not combined to another molecule. Regardless of the multiplicity of infection the same amount of polysaccharide was produced by each infected cell at any one time and was independent of the numbers of elementary bodies formed. This finding might indicate that the polysaccharide is a product of the host cell; but recent work by Jenkin & Fan (1971) on the preferential utilization of UDP-glucose by the glycogen synthetase of the mammalian host cell and of ADP glucose by Group A *Chlamydia* suggests that it is made by the organism.

Although Gill & Stewart (1970) found that the amount of glucose in the culture medium limited production of infective psittacosis organisms, their greatest concentration of glucose was 100-fold less than that used in experiments reported here, in which yields of polysaccharide were not increased when the concentration was doubled.

Bernkopf, Mashiah & Becker (1962) suggested that the polysaccharide might function as an energy reserve, 'used up during the final stages of maturation of the agent'. This hypothesis was based on the finding that the proportion of inclusions staining brown with iodine decreased between 28 and 48 hr. after infection, as the numbers of infective particles increased. By contrast, Reeve & Taverne (1967) found that the proportion of inclusions staining with iodine continued to increase after 28 hr. to reach a maximum at least 48 hr. after infection. The ability of inclusions to be stained with iodine reflects to some extent their content of polysaccharide, but neither of these groups of workers measured this compound in inclusions.

Precise chemical measurements revealed a maximum 48 hr. after infection, about 20 hr. after first appearance of infectivity. There is no obvious explanation for the discrepancy between these results and those of Bernkopf *et al.* (1962), especially since in terms of infectivity the growth cycles were closely similar.

According to the suggestion made by Bernkopf and his colleagues, the decrease in amount of polysaccharide per inclusion during the later part of the growth cycle results from its biochemical degradation within the inclusion. However, the results reported here show that substantial amounts of polysaccharide appear in the medium of infected cultures. It is unlikely that this amount of free polysaccharide resulted from rupture of infected cells since it would be necessary to postulate that half the cells had lysed 30 hr. after infection to account for it, a notion not supported by microscopical examination of the cultures. The decrease in polysaccharide content of infected cells between 48 and 56 hr. after infection could be entirely accounted for by the observed rate of leakage of 10 μg. per hour.

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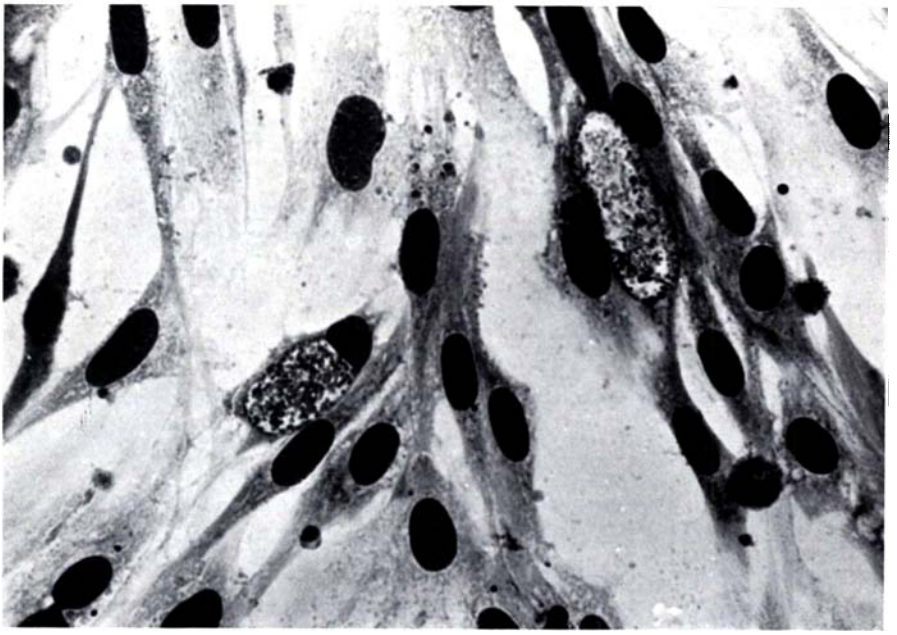
EXPLANATION OF PLATE

Fig. A. BHK-21 cells 45 hr. after infection with < 1 TRIC organism per cell. (× 480.)

Fig. B. BHK-21 cells 45 hr. after infection with 12 TRIC organisms per cell. (× 480.)



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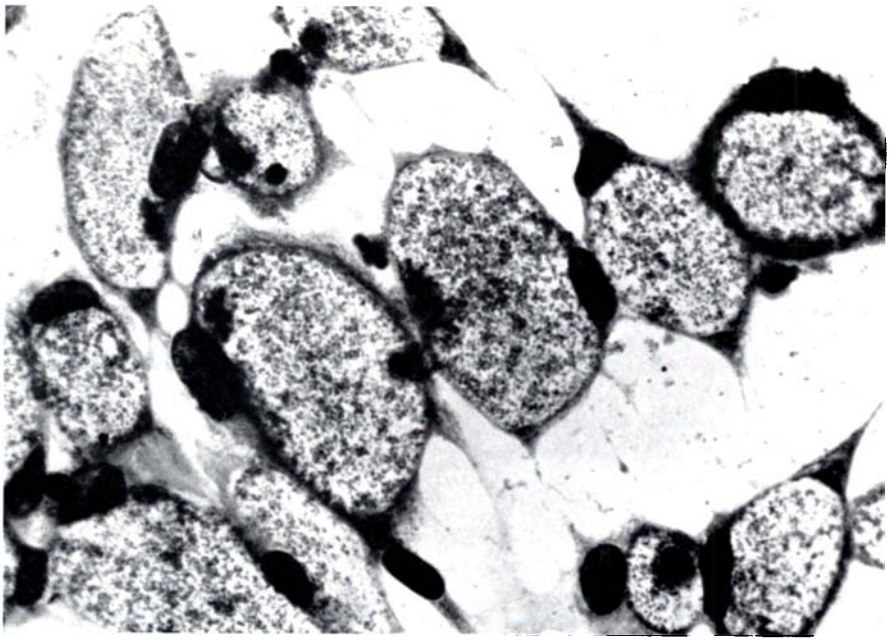

B

ANDREA EVANS

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