A NEGATIVE STAINING METHOD FOR HIGH RESOLUTION ELECTRON MICROSCOPY OF VIRUSES

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SUMMARY

A simple technique has been developed for the study of the external form and structure of virus particles. High contrast with good preservation is obtained by mixing virus preparations with 1 % phosphotungstic acid adjusted to pH 7.5 and spraying directly onto electron microscope supporting films made from evaporated carbon. The application of the technique to tobacco mosaic virus and turnip yellow mosaic virus is described. Structural details suggested by X-ray diffraction methods have been resolved.

INTRODUCTION

Electron microscopical observation of virus particles offers the possibility of giving direct information about their external form and structure. Instruments with extremely high resolving power are available but there are major limitations in the techniques of specimen preparation[1]. The introduction of the critical-point method[2] and freeze-drying[3] improved the preservation of three-dimensional morphology but the problem of attaining sufficient contrast, without obscuring structural detail, has not been completely solved. Metal shadowing gives information about the size and shape of viruses but it does not reveal internal structure and often obscures surface regularities. It also enhances the roughness of the substrate film which may make detailed observation of small viruses difficult. The study of virus particles in ultra-thin sections of pellets or infected cells raises problems of fixation artefacts and there is often ambiguity in interpreting the contrast produced by the stains used.

In this paper, we wish to describe a technique which extends the range of electron microscopical study of virus structure. It consists very simply in "embedding" the virus particles in an electron dense material, in this case, potassium phosphotungstate, which introduces contrast by negative staining. The potentialities of this technique have been previously recognised by HALL[4] and by HUXLEY[5]. When bushy stunt virus particles were treated with phosphotungstic acid and the stain incompletely removed by washing, HALL found that better definition of the particles was often obtained by outlining them by the stain. HUXLEY showed that it was possible to reveal the central hole in tobacco mosaic virus by what amounted to the same procedure. We have been able to define exact conditions for the routine use of this technique. Under

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these conditions, the phosphotungstate has never been observed to stain directly either the protein or the nucleic acid of virus particles which remain relatively electron transparent. If there are any cavities within the virus which are accessible to the phosphotungstate, these will be revealed and the technique is thus capable of disclosing internal structure. We have used it most successfully in a recent study of bacteriophage structure where it could be shown that in addition to the high contrast available there was excellent preservation of external form[6]. The application of the technique to tobacco mosaic virus and turnip yellow mosaic virus will be described in this paper. These viruses were chosen as representative of a rod-shaped and small spherical virus respectively and also because their structure has been intensively studied by electron microscopy, X-ray diffraction and other physico-chemical methods.

MATERIALS AND METHODS

Before describing the technique for negative staining, it is of importance to discuss the methods used for preparing the substrates necessary for specimen supports. Nitrocellulose and formvar films were found unsuitable due to thermal drift in the electron microscope. It was further noted that this type of substrate frequently ruptured immediately after spraying these particular virus preparations.

Evaporated carbon, stripped from mica surfaces was found to be sufficiently stable and rigid to support the droplet patterns[7,8]. Freshly cleaved mica, cut to the size of microscope slides, was placed in an experimental evaporator using well trapped mercury pumps[9]. Carbon was then evaporated onto the cleaved mica surface from a distance of approximately 20 cm. The estimated thickness of the films appeared to be of the order of 150–200 Å. By slowly immersing the coated mica into water, a sheet of carbon can be floated onto the surface. Electron microscope grids are placed below the carbon film and raised slowly, resulting in the entire grid area being covered with the carbon substrate.

Carbon substrates prepared in evaporator plants, using oil pumps, frequently caused the spray droplet patterns to form dense spherical areas with little evidence of the spreading effect produced on carbon films from Hg units. The possibility of "backstreaming" oil vapour onto the carbon surface may result in different surface conditions being unsuitable for spraying techniques.

Phosphotungstic acid preparations

A 2 % solution of phosphotungstic acid was made up and adjusted to pH 7.4 using normal KOH. To 1.0 ml of virus suspension in water or ammonium acetate is added 1.0 ml of PTA, and the solution poured into a commercial atomiser spray. The type used in these experiments was a Vaponefrin glass unit with a normal hand bulb. The solutions are then sprayed directly onto the carbon-coated grids. It was found necessary to adjust the concentration of each virus preparation. Droplet patterns containing low concentrations of virus tend to remain as dense opaque spheres, whereas ideal concentrations produced relatively large thin areas with particles clearly visible.

Specimens were examined in the Siemens Elmiskop electron microscope and photographed at instrumental magnifications of 40,000 to 80,000 ×. All the electron

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micrographs were taken using the double condenser lens system producing an illuminating beam of approximately 10–20 μ in diameter. Single condenser lens illumination or broad beam conditions frequently resulted in heating the droplet pattern causing serious distortion and decomposition effects in the PTA. Microdiffraction patterns taken from droplet patterns containing PTA suggested that the dense areas were amorphous.

From measurements made during investigations of the components of bacteriophage, structures approaching 15 Å or less could be resolved[6]. The limitations in terms of resolving power are difficult to estimate as much of the structure below 15 Å is confused by phase-contrast effects and contamination.

RESULTS AND DISCUSSION

Tobacco mosaic virus

Fig. 1 is a low power micrograph of TMV prepared by the phosphotungstate method. The rod-shaped viruses are clearly outlined by the electron dense background and, even at this magnification, the axial hole running down the centre of the rod can be readily seen. A high power micrograph is shown in Fig. 2. The rods are generally 150 Å in diameter and the mean diameter of the axial hole is 35–40 Å. The size of this hole agrees well with that found by FRANKLIN AND HOLMES[10] using X-ray diffraction. Although the inter-particle distance in dry orientated TMV is found to be 152 Å by X-ray diffraction it has been shown that the diameter of the particle is greater than this and could be as much as 180 Å. The radial density distribution shows a strong maximum at about 78 Å and FRANKLIN AND KLUG[11] reached the conclusion that while the *mean* diameter of the particle is 154 Å, the particle is deeply furrowed by a helical groove which follows the helical arrangement of the protein subunits. In dry orientated preparations the particles interlock to give the interparticle distance of 152 Å.

We have been unable to reveal the helix with the phosphotungstate method. It would appear that the protein surrounding the helical grooves is occluded by the phosphotungstate, which explains why the observed particle diameter is only 150 Å. Further evidence for this is provided by the finding that parallel particles are never seen to interlock and the closest distance measured between centres of adjacent particles is 170–180 Å. In this case therefore the interparticle distance is a reflection of the *maximum* diameter of the particles, while the observed diameter reveals that part of the protein which is impermeable to the phosphotungstate.

A comparison of the radial density distribution of the intact protein and of repolymerized protein lacking RNA, showed a density maximum at radius 40 Å in the virus to be replaced by a density minimum in the nucleic acid-free protein[10]. This suggested that the RNA followed a helical path through the length of the particle at a radius of 40 Å (see also HART[12]). We have attempted to display the cavity occupied by the RNA in the following way. Nucleic acid-free protein was prepared from TMV using the acetic acid procedure of FRAENKEL-CONRAT[13]. At pH 7 such solutions were clear and when observed in the electron microscope showed no visible structures. After dialysis against 1 % ammonium acetate adjusted to pH 5 with acetic acid the solution became opalescent, and a micrograph of a PTA preparation is shown in Fig. 3. Rods of varying length can be recognised but their structure

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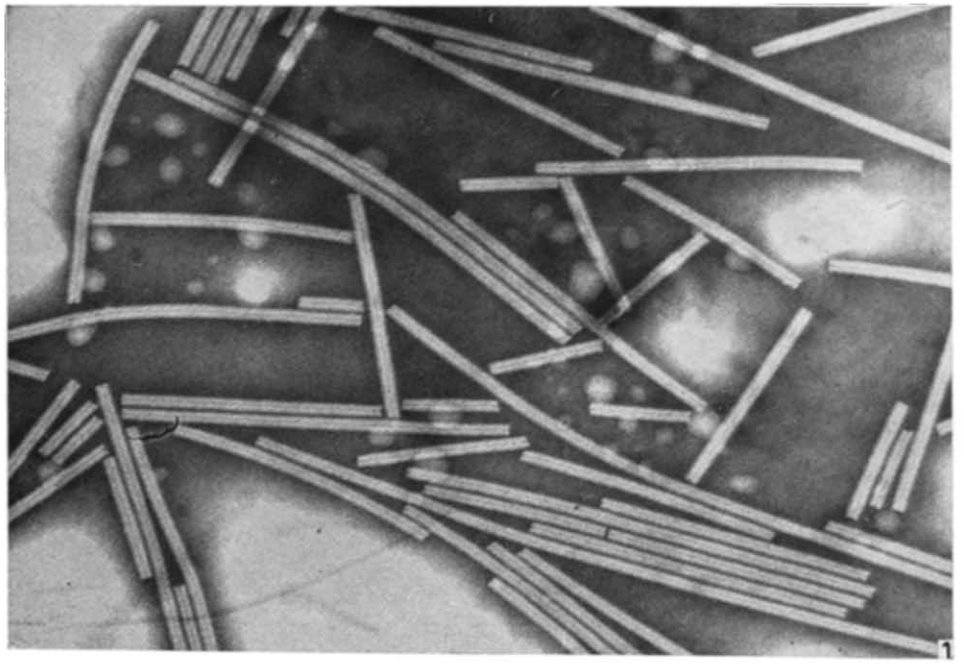


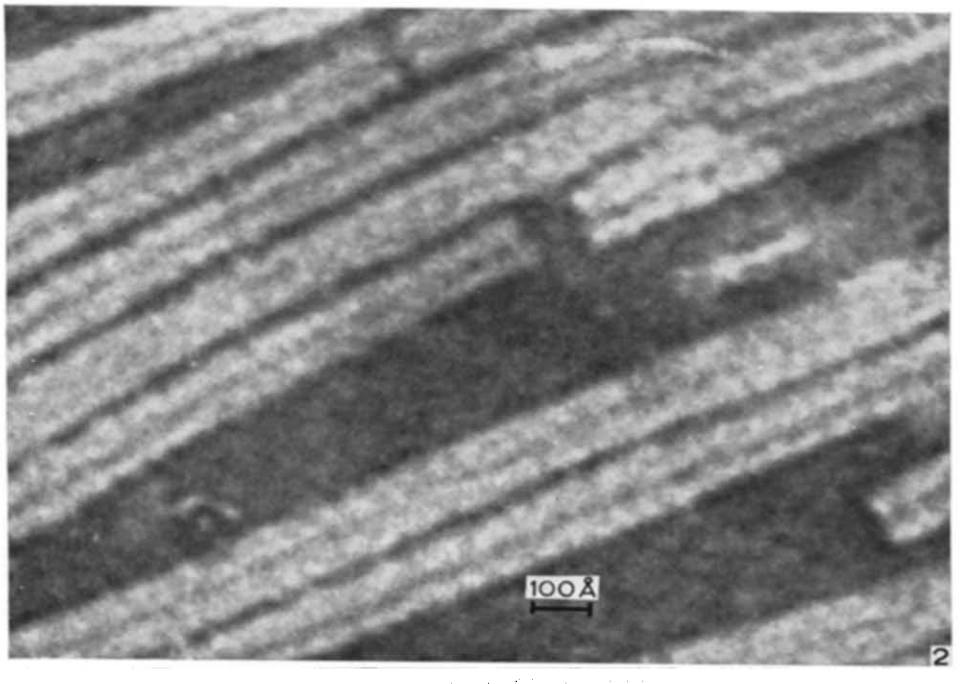
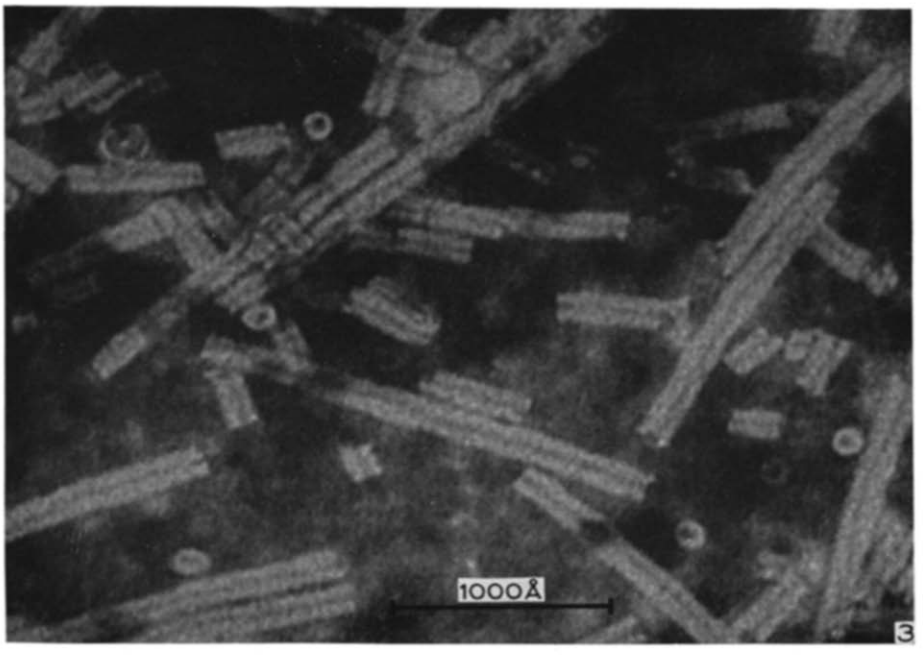


Fig. 1. Low magnification of droplet pattern area showing tobacco mosaic virus rods "embedded" in phosphotungstic acid. No staining of the protein is evident. 59,000 ×.

Fig. 2. TMV rods showing penetration of PTA along central region of virus rods. 645,000 ×



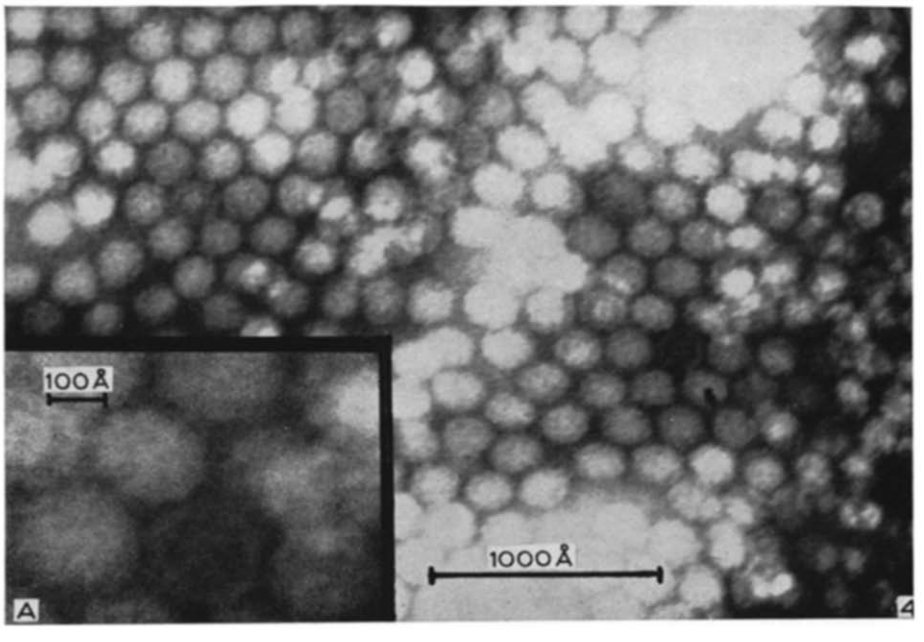
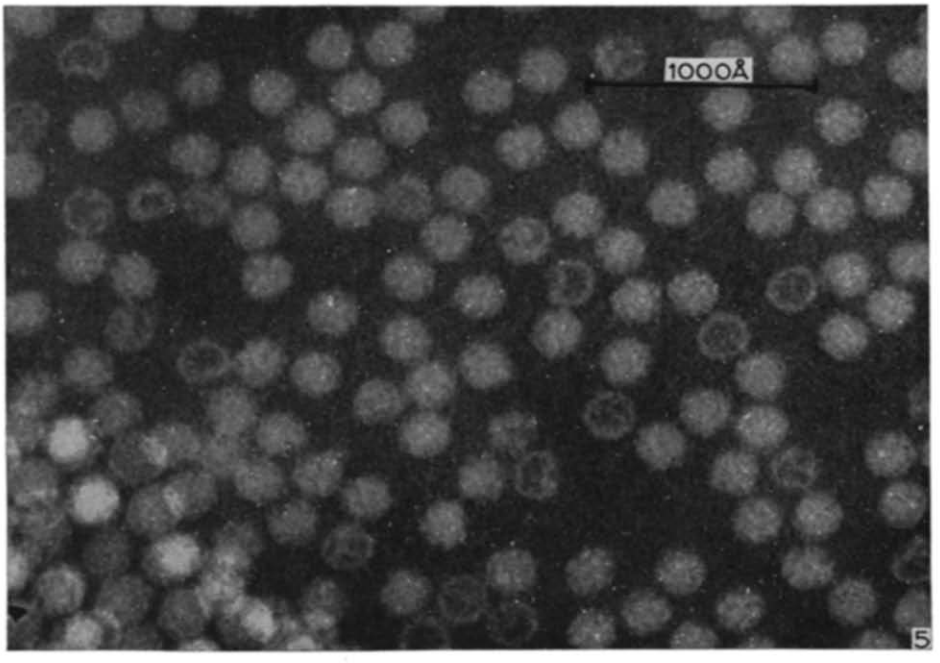


Fig. 3. Reaggregated nucleic acid-free protein. A number of particles can be seen as hollow discs with central hole diameters of 70–80 Å. 252,000 ×.

Fig. 4. Turnip yellow mosaic virus crystallayer showing internal structure with particles in typical hexagonal close packing. 256,000 ×.

Fig. 4a. Enlargement showing empty protein shell packed in the structure. 645,000 ×.



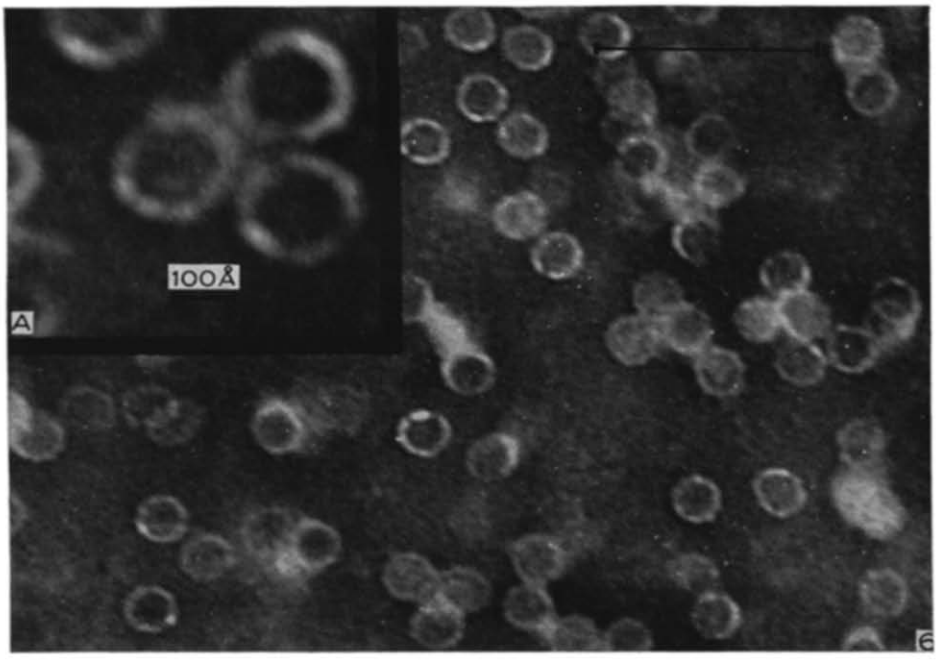


Fig. 5. Isolated TYMV particles from preparations dialysed against distilled water. The structural detail appears as a granularity. Empty particles may also be seen. 254,000 ×.

Fig. 6. Empty protein shells in top component of TYMV showing little evidence of granularity. 254,000 ×.

Fig. 6a. Enlargement of a group of these "ghosts". 645,000 ×.

appears to be more disorganised than the intact particles. All rods show the central hole of 40 Å diameter again confirming the X-ray diffraction results. There is, however, no indication of any increased density at a radius of 40 Å. In addition to particles lying on their sides, the micrograph also shows particles lying on their ends. These discs show a central hole which is much wider than 40 Å. Measurements show the average diameter to be 70–80 Å. It is possible that, in this case, the increase in diameter of the hole is due to PTA filling up the helical tunnel occupied by RNA and that we are unable to resolve the 20 Å of protein between the central hole and the helical tunnel. In particles in this orientation the density of the PTA would be summed over a number of turns of the helix and hence sufficient contrast might be available which would not be the case in particles lying on their sides.

Turnip yellow mosaic virus

When a suspension of crystals of this virus in half saturated ammonium sulphate is rapidly diluted fifty times with 1 % ammonium acetate and prepared by the phosphotungstate method, regularly packed virus particles can be observed in the electron microscope (Fig. 4). This arrangement is due to fragments of crystals and is not produced by rafting of individual particles as can be readily shown by preparing dialysed solutions of the virus at the same concentration. In these crystal layers the particles are hexagonally close packed and each particle appears to be polygonal in shape. The diameter of the virus is 210 Å, and the interparticle distances 240 Å, which is to be compared with the diameter of 280 Å estimated by X-ray diffraction of crystals[14]. It should be mentioned that, in such preparations, the ammonium sulphate does not interfere with observation since it crystallizes on drying and areas free of crystals are frequently found.

Isolated particles may be seen in preparations made from crystals dialysed against distilled water as shown in Figs. 4 and 5. The polygonality of the particles is not as marked as in the intact crystals, but many particles show structural detail. This appears as a granularity and is reminiscent of the structure found by STEERE[15] in frozen-replica preparations. In both the crystal and isolated particle preparations "ghost" particles can often be seen (Figs. 4 and 5). These are clearly particles without nucleic acid and are therefore the empty protein shells characteristic of the so-called top component[16]. Figs. 6 and 6a are electron micrographs of a purified preparation of top component, which shows most of the particles to be empty. The thickness of the protein membrane in such particles is 20–25 Å; this should be compared with the value of 35 Å estimated by X-ray diffraction. Such empty particles never show the granular appearance of the complete virus and it is possible that this reflects some organisation of the RNA in the particle.

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References p. 110.

REFERENCES

1 R. C. WILLIAMS, *Advances Virus Research*, 2 (1954) 184.
2 T. F. ANDERSON, *Trans. N.Y. Acad. Sci.*, II, 13 (1951) 130.
3 R. C. WILLIAMS, *Exptl. Cell. Research*, 4 (1953) 188.
4 C. E. HALL, *J. Biophys. Biochem. Cytol.*, 1 (1955) 1.
5 H. E. HUXLEY, *First European Conference on Electron Microscopy, Stockholm, 1956*, p. 260.
6 S. BRENNER, G. STREISINGER, R. W. HORNE, S. P. CHAMPE, L. BARNETT AND S. BENZER, *J. Mol. Biol.*, 1 (1959) in the press.
7 D. E. BRADLEY, *Brit. J. Appl. Phys.*, 5 (1954) 65.
8 C. E. HALL AND M. LITT, *J. Biophys. Biochem. Cytol.*, 4 (1958) 1.
9 V. E. COSSLETT AND R. W. HORNE, *Vacuum*, 5 (1955) 109.
10 R. FRANKLIN AND K. C. HOLMES, *Acta Cryst.*, 11 (1958) 213.
11 R. FRANKLIN AND A. KLUG, *Biochim. Biophys. Acta*, 19 (1956) 403.
12 R. HART, *Biochim. Biophys. Acta*, 28 (1958) 457.
13 H. FRAENKEL-CONRAT, *Virology*, 4 (1957) 1.
14 A. KLUG, J. T. FINCH AND R. FRANKLIN, *Biochim. Biophys. Acta*, 25 (1957) 242.
15 R. L. STEERE, *J. Biophys. Biochem. Cytol.*, 3 (1957) 45.
16 R. MARKHAM, *Discussions Faraday Soc.*, 11 (1951) 221.

ALTERATIONS IN THE RADIOSENSITIVITY OF *ESCHERICHIA COLI* THROUGH MODIFICATION OF CELLULAR MACROMOLECULAR COMPONENTS

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SUMMARY

The number of survivors of *E. coli* strains B/r or 15_T^- following X-ray exposure was increased if the log phase cells had been previously grown in the presence of chloramphenicol. By omission of thymine in the growth medium of *E. coli* strain 15_T^- the chloramphenicol effect failed to materialize. Under these conditions net DNA synthesis was prevented while RNA continued to be accumulated. Since protein synthesis was inhibited by chloramphenicol under all conditions employed it would appear that the "surplus" DNA was responsible for the enhanced survival of cells so treated.

INTRODUCTION

While numerous investigations have been made on the relative roles of the nuclear and cytoplasmic nucleic acids in radiosensitivity of animal and plant cells, similar information on bacterial cells has been difficult to obtain because of the refractory nature of such cells to the usual physical manipulations found successful with the larger cells. Recently, however, several means of altering the ribonucleic acid (RNA), deoxyribonucleic acid (DNA), and protein (P) contents of bacteria have been described[1,2]. In the present work alterations in macromolecular components have been

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