THE USE OF RADIOAUTOGRAPHY AND ELECTRON MICROSCOPY FOR THE LOCALIZATION OF TRITIUM LABEL IN BACTERIA

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Localization of tritium label in bacteria has been successfully demonstrated both at the intercellular level (1) and at the intracellular level (2). However the use of Kodak AR-10 (3) stripping emulsion gives rise to a complication in those experiments. The 2 μ maximum range (4) of the β-rays from tritium in this emulsion, plus the greater than 2 μ thickness of the stripping emulsion itself, combine to allow a single point source to give photographic grains frequently located as far apart as 2.5 μ. Since this distance corresponds to the length of a large *E. coli* cell, one cannot associate any grain pattern above a bacterium, or section of a bacterium, with any one portion of the specimen.

To improve on the physical localization of label in whole cells, one might select a tracking emulsion, *i.e.* an emulsion giving photographic grains closely spaced along the paths of the particles, in this case the weak β-rays from tritium atoms. One would then be able to trace each track to its origin (5). Alternatively, one might confine the photographic grains to a region near the point of origin of the emanating radiations by using thin films (9, 10). It is the latter approach which is utilized in this paper.

From considerations of solid angle a fine grain emulsion (6) of 0.2 μ thickness should give localization to well within 1 μ. It was found that Kodak V-1055, a non-tracking, x-ray gel emulsion similar to Kodak AR-10, can be easily converted to a thin film. With films of this emulsion, improved localization can be realized even for observations using the light microscope. Additional improvement can be achieved by viewing the radioautograph in an electron microscope. The improved optical resolution and the increased depth of field of the electron microscope (7) not only give better visualization of the structure under examination but facilitate the identification of the individual, small (<0.2 μ) and often adjacent photographic grains. The appearance of such photographic grains in the electron microscope has already been described by Hall and Schoen (8).

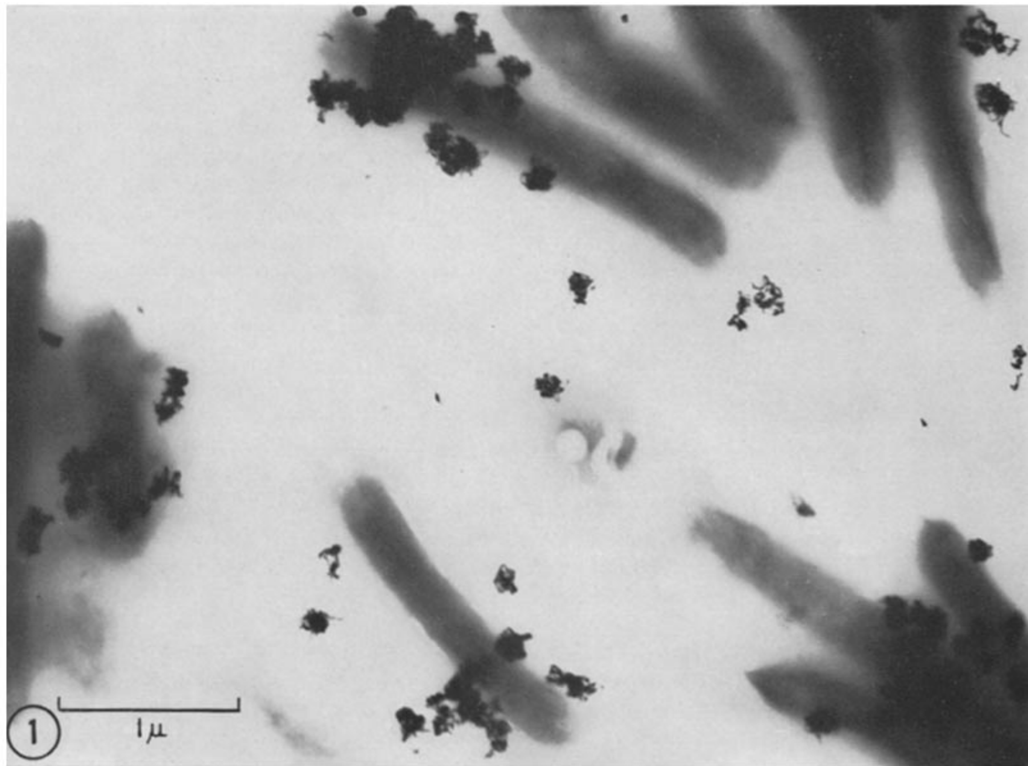
The first work combining electron microscopy and radioautography is due to Liquier-Milward (5). By using a tracking emulsion he was able to demonstrate the *in vitro* incorporation of Co^{60} into mammalian nuclei. Subsequently George and Vogt (10), using thin film tracking emulsion, were able to identify Pu^{239} labeled dust particles. Similarly, O'Brien and George (9) by examining thin sections of individual yeast cells and superimposing thin photographic films localized adsorbed Po^{132} on the cell walls.

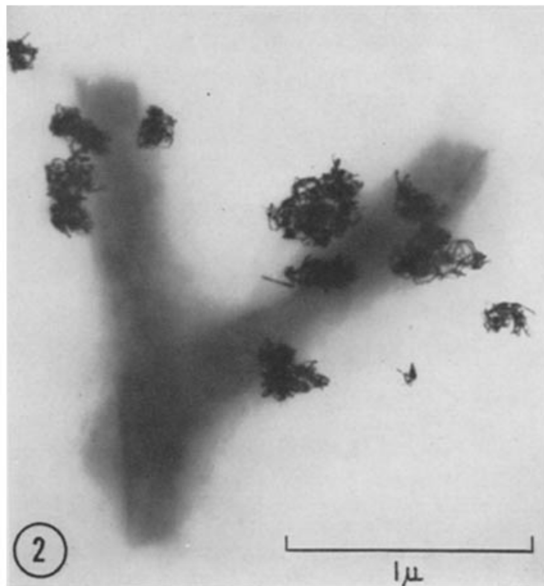
In the work presented here the localization of DNA labeled with thymidine-H^3 (11–13) was studied in *E. coli* three generations after the labeling had taken place (1). The method used was to examine in the electron microscope radioautographs of the cells using a thin film of Kodak V-1055 x-ray emulsion. In so doing it was possible to localize the labeled DNA in whole bacteria to regions <1 μ in length and $<\frac{1}{2}$ μ^2 in area.

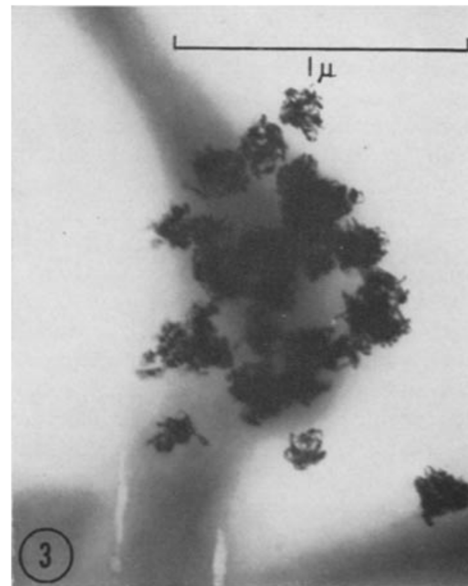
MATERIALS AND METHODS

Cells of *E. coli* 15 T^- (14) were grown at 23°C. for ten generations in Anderson's (15) M-9 plus 0.4 per cent glucose medium supplemented with 4 μg./ml. tritium-labeled thymidine (Schwarz Labs, Inc., 3 c/mM) purified further by paper chromotography (16). The cells grew to the resting stage. At that time some cells, hereafter referred to as "fully labeled cells," were removed from the medium and fixed in 2 per cent formalin (17). The remaining cells were diluted by a factor of 10 into fresh medium containing 0.04 per cent unlabeled thymidine. The cells then divided about 3.2 times; *i.e.*, until they exhausted the medium and reached the resting stage. Such cells are subsequently called "third generation cells." They were fixed in 2 per cent formalin for 1 day, and centrifuged in distilled water to remove the remaining formaldehyde and radio-thymidine.

The autoradiographic method of George and Vogt







FIGURES 1 to 3

Electron microscope pictures of radioautographs of cells of *E. coli* 15 T^- labeled with thymidine-H^3 and subsequently grown for three generations. Fig. 1, × 24,000. Fig. 2, × 33,000. Fig. 3, × 39,000.

(10) was modified by gluing Scotch tape strips, with sticky surface up, to glass microscopic slides. Copper grids, 300 mesh per inch, which had previously been coated with formvar (18) and shadowed with carbon, were gently placed on the Scotch tape. Cells were pipetted onto the coated grids and allowed to dry. Using a Wratten series "I" for safe-light illumination, the samples were coated with V-1055 emulsion. This was accomplished by placing a loop full of sol emulsion directly above the sample, waiting and thereby allowing gelling to occur, and then gently blowing down. The emulsion coating was dried for 1 hour at 27°C.

The samples were stored in CO_2 at 4°C., 15 per cent relative humidity (19), for 6 days. They were developed at 20°C. for 1 minute in Kodak D-19b developer, placed ¼ minute in acid stop bath, introduced for 2 minutes to acid fixer, and washed gently for 4 minutes in distilled water. The grids were thoroughly dried and removed (also, gently) from the Scotch tape and then examined in a Siemens model 7 electron microscope.

RESULTS AND DISCUSSION

Where cells of *E. coli* 15 T^- are labeled with thymidine and allowed to grow for about three generations, as described in the methods, approximately half the cells show radioactivity. Figs. 1 to 3 show electron micrographs of such radioautographed cells. The silver, ribbon-like grains and the bacteria are both clearly visible. It will be noted that labeling is present in only half of a cell. These cell regions are $<1\ \mu$ in length and $<\frac{1}{2}\ \mu^2$ in area. Over one-third of the labeled third generation cells show this particular localization. Approximately one-third of the labeled cells show a concentration of grains in the central region while less than one-third show a random grain pattern. In contrast fully labeled cells show a consistently random grain pattern. The random patterns could be of biological significance. At the same time, they could reflect a slight shifting of the grains in the washing process. Localized thick regions of the photographic emulsion would also prevent the localization of label; therefore in working with this emulsion certain precautions should be followed: (*a*) To assure a minimum of shifting and folding of the photographic film, use minimum times for the developing process. (*b*) To assure a uniform film, particularly in the regions near the bacteria, allow the film to gel before application to the sample.

The sensitivity of the film is between one-fifth and one-half that of Kodak AR-10 emulsion, therefore the thickness of the film is estimated to be between 0.2 μ and 0.4 μ. With improved control of film thickness and film uniformity more quantitative results should be forthcoming. Nevertheless, Figs. 1 to 3 do show that it is possible to localize DNA structures in parts of a bacterial cell.

Such DNA structures have been described by Forro and Wertheimer (20) who demonstrated very convincingly that these structures replicate in a semiconservative manner analogous to the behavior of DNA at the molecular level (21, 23) and at the chromosomal level (22). Non-randomness for the separation of four main DNA structures in *E. coli* has also been statistically demonstrated (24). The demonstration reported here of the localization of each DNA structure in one-half of the cell, taken with the above demonstrations, supports the view that the chromosomes in *E. coli* are haploid (25), two in number (26), and physically separated (27). These results will be discussed in more detail elsewhere (28).

By using Kodak V-1055 emulsion it has been shown that electron microscopy and radioautography can be simply combined for examining tritium labeled bacteria. The localization of label to areas of the order of less than $\frac{1}{2}\ \mu^2$ should commend this approach to investigators wishing to examine high specific activity, tritium labeled biological preparations.

The planning of the experiment reported here was greatly aided by the information contained in the published experiments of others. I am particularly grateful to Mr. G. Hills, for generously interrupting his work to perform the electron microscopy. I should like to thank Mr. S. Frey, Mr. I. Flack, Miss V. Munoz, and Miss Y. Carter for able technical assistance and Dr. R. Markham for valuable discussions and criticism.

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