DIRECT STAINING OF THE TWO TYPES OF NUCLEOPROTEINS IN ESCHERICHIA COLI

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This report describes a technique for the differential staining of the two types of nucleoproteins in bacteria. The staining procedure is adapted from that described by Jacobson and Webb (1952) for other cells. In several respects it is similar to techniques previously used in bacterial cytology. Visualization of the two nucleoproteins individually is possible after treatment with specific nucleases.

MATERIALS AND METHODS

Basic staining procedure. In this procedure ribonucleoprotein is stained blue and desoxyribonucleoprotein is stained red.

(1) Impression smears are made on alcohol cleaned coverslips from squares cut out of agar plates on which bacterial cells are grown directly on the agar surface or are placed there from liquid culture and retained until the excess moisture has soaked into the agar.

(2) The coverslip preparations are immersed immediately in absolute methanol previously chilled on dry ice (Blank et al., 1951) and left on dry ice overnight.

(3) Coverslips are placed in absolute methanol at room temperature for a few seconds. They are transferred to a 1:5 dilution in methanol of a saturated methanol solution of methylene blue eosinate and stained for 10 minutes.

(4) Coverslips are rinsed quickly in distilled water (adjusted to pH 7.0 with NaOH) to rehydrate and wash off excess dye.

(5) Preparations are placed in freshly prepared aqueous Giemsa solution (1:10 dilution, pH 7.0) for 10 minutes.

(6) The excess dye is removed from the coverslips by a brief rinse in distilled water, pH 7.0.

(7) The coverslip preparations are dehydrated rapidly by 4 quick dips in acetone at room temperature, 4 dips in a 1:1 mixture of acetone and xylol, and then transferred through two changes of xylol before draining (blotting should be avoided), and mounted on alcohol cleaned glass slides. Final dehydration may be achieved also through the use of overnight dehydration in acetone previously chilled and maintained on dry ice; the coverslips then are immersed a few seconds in acetic acid at room temperature before the transfers through xylol.

Reagents used. It is recommended that all reagents be replaced after several hours use.

(a) Fixative. Fixation in absolute methanol at room temperature frequently results in extreme shrinkage, in “budding” cells, or vacuolization (figure 1, a–c). The use of cold methanol keeps the production of these gross fixation artifacts to a minimum although limited shrinkage and slight peripheral vacuolization of the cells still occur in some preparations. Also, where cells are spread thickly, distortion by adjacent cells may result (figure 1, d).

A number of agents were used for prefixation of specimens prior to immersion in methanol. All produced extensive shrinkage at least in some areas of the slide. Better results with alcohol containing reagents were obtained when fixation was carried out at –5 C (Wolman and Behar, 1952) rather than at room temperature. None of the methods gave as good preservation of the cells as did absolute methanol cooled on dry ice.

(b) Methylene blue eosinate. Saturated solutions of May-Greenwald stain and Leishman’s stain were made up in absolute methanol and “aged” for one week or more prior to use.

(c) Giemsa stain. The Giemsa stain or various substitutes were diluted 1:10 in distilled water

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2 Methanol, Baker analyzed reagent, ACS.


4 Coleman Bell and Co., Norwood, Ohio.
immediately before use. A ready-made solution (Gradwohl Laboratories, Cert. no. GGe-13) and a solution prepared from powder (Giemsa stain, original azure blend type, Cert. no. LGe-16) were satisfactory.

(d) Mounting media. Harleco synthetic resin (HSR) was used in most of the work, but "technicon" and "clarite" mounting media likewise were found to be suitable. In Canada balsam, pronounced fading occurred in a few days. Although fading was generally unequal throughout the slide, the methylene blue eosinate was always the first to fade, revealing the Giemsa stained material with clarity. Most preparations mounted in Harleco synthetic resin do not fade (during a period of a year or longer); in those few areas where fading is detectable, the blue stain has left the cytoplasm and has become adsorbed to the nuclear structure.

(e) Enzymes. Preparations were immersed in 0.4 mg per ml ribonuclease in distilled water at pH 6.0 or 6.8 for 15 minutes or longer at 37 C after fixation with cold methanol or with Chabaud's solution. Osmium tetroxide fixation greatly inhibited ribonuclease action. Two samples of crystalline ribonuclease (General Biochemicals and Armour and Company) gave identical results.

Absolute methanol fixed coverslip preparations were exposed one hour to crystalline desoxyribonuclease (Worthington) according to the procedure of Murray and Whitfield (1953). Similarly fixed specimens were exposed to 0.4 mg per ml crystalline lysozyme (Delta) in pH 6.6. Diluent controls were included with each series.

Optics and photomicrography. Bausch and Lomb light and phase contrast optics with 12.5 X or 15 X eyepieces and 97 X objectives were used. Photographs were taken on Kodak Micro-film 35 mm and Kodak Panatomic X film. Filters, to increase contrast in the desired stain, were used in the tungsten filament and zirconium arc lighting systems. One set of filters consisted of either Kodak written red filters A25 and F29 or Bausch and Lomb interference filter 630 m, while the other set included either written blue filter H45 and green filter B58 or Bausch and Lomb interference filter 520 m.

Organisms studied. Most of the studies were performed with log phase broth cultures of Escherichia coli, strain B. However, the standard staining procedure has been applied to 18 hour

All figures are reproduced here at a final magnification of 3,675 X. All preparations are of uncrushed specimens and represent log phase broth cultures of Escherichia coli, strain B.

Figure 1. Stained by complete procedure described in Methods and Materials and photographed to show the staining throughout the cell. (a) Extreme shrinkage, often found in preparations fixed in methanol at room temperature, (b) "budding" cell, found in preparations fixed in methanol at room temperature, (c) vacuolization, found mainly in cells fixed at room temperature but also, occasionally in those fixed in cold methanol, especially if air drying is not avoided, (d) distortion of adjacent cells, occasionally found in the more thickly spread areas of the coverslip.

Figure 2. Living cells placed on agar from a log phase broth culture and photographed with phase contrast.

Figure 3. Cells fixed in cold methanol, placed in Leishman's stain for 10 minutes, rinsed in distilled water, dehydrated through acetone and xylol and mounted in Harleco synthetic resin (HSR). The cytoplasm is stained blue; nuclei are unstained or only very faintly stained blue.

Figure 4. Stained by the Bouin-thionin procedure (Murray et al., 1950).

Figure 5. Stained by the complete procedure described in Methods and Materials. Photographed to show the blue cytoplasmic staining. However, as comparison with figure 3 will show, the optical density of the nuclear staining (red) prevents visualization in black and white photographs of the nuclear areas.

Figure 6. Same cells as figure 5, this time photographed to show more clearly the red nuclei. Again, the contrasting stain cannot be removed completely by the use of filters.

Figure 7, a - c. As figure 6, except treated with ribonuclease prior to staining.

Figure 8, a, b. As figure 6. Preparations in which some fading of the cytoplasm has occurred. Photographed to show nuclear structure.

Figure 9. Piekarski-Rabinow nuclear stain prepared as described in Murray and Whitfield (1953) and photographed in wet mount. Hydrolyzed in 1 N HCl at 60 C for 8 minutes.

Figure 10, a, b. DeLamater (1953a) azure A-thionyl chloride stain after fixation in vapors of 2 per cent osmium tetroxide for one minute. Hydrolyzed in 1 N HCl at 60 C for 8 minutes.

Figure 11. As figure 10, except fixed overnight in cold methanol and rehydrated before hydrolysis.
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Figs. 1–11
and log phase cultures of 14 representative gram negative genera. Although other gram positive genera were examined, the organism used for most of the experiments involving a gram positive organism was the strain of Bacillus megaterium used by DeLamater and co-workers.

RESULTS

Figure 2 shows living log phase E. coli, strain B, cells photographed with the phase contrast microscope. Staining with methylene blue eosinate produces blue cells with intensely stained polar and central areas and lighter regions intermediate (figure 3). This distribution of stain is similar to that produced by the Bouin-thionin procedure for pentosenucleic acid (compare with figure 4) but more closely resembles the gradation of densities observed in the living cells. The areas of lesser density are the sites of the bacterial nuclei.

The completed staining process is shown in figure 5. The cells are photographed to show the localization of eosinated methylene blue stained material in the cytoplasm although the optical density of the Giemsa stained component prevents an adequate demonstration by black-and-white photographs. Cell size and shape are preserved by the methanol fixation.

Ribonuclease completely removes the methylene blue eosinate-staining substances (figure 7, a-c). These are removed also by alkaline extraction with Na2HPO4 at 60 C for 3 hours (Welsch and Nihoul, 1948). Nuclear areas take on a red coloration with Giemsa and fail to stain with the eosinated methylene blue. The cytoplasm also loses its staining affinity after hydrolysis in 1 N HCl at 60 C for 7 to 8 minutes; however, in this case, unlike the previous treatments, the nuclei now stain with both eosinated methylene blue and Giemsa.

Figure 6 shows cells photographed with filters enhancing the contrast between the red Giemsa stained material and the blue cytoplasm. While nuclei may be discerned in these cells, their fine structure is obscured in part by the cytoplasmic staining. Finer details of nuclear structure are visible after ribonuclease digestion (figure 7, a-c) and cytoplasmic fading in situ (figure 8, a, b). The nuclei are composed of discrete elements with chromosomal configurations. Small extranuclear granules (corresponding to the centrioles of DeLamater, 1953a, b, c) are present near some nuclei. The centrioles are found adjacent only to those nuclei which have configurations characteristic of dividing stages, as if they became achromatic or much smaller during other nuclear stages.

Nuclear staining by the Feulgen technique (as modified by Beutner et al., 1953) is too faint to allow definition of fine structure. On the other hand, in Piekarski-Robinow wet mounts (figure 9) the intense nuclear staining hinders study of the nuclear components as entities. DeLamater staining with either osmium (figure 10, a, b) or methanol (figure 11) fixation shows the chromosomal elements more clearly.

During infection of E. coli with T2 bacteriophage, the distribution of the Giemsa stained material undergoes relocalization, in accord with that previously reported for the desoxyribonucleic acid of infected cells (Murray et al., 1950; Beutner et al., 1953).

Finally, exposure of the cells to desoxyribonuclease eliminates the Giemsa stained substance but does not alter the staining of the cytoplasm with the methylene blue eosinate.

Metaphosphate (volutin) stains red with the basic staining procedure in those genera which contain large amounts of this inclusion, e.g., Corynebacterium diphtheriae, Mycobacterium spp, Spirillum spp. The red coloration of metaphosphate, in addition to the usual methods for its characterization, differs from the red nuclear staining in three respects: (1) it is produced by methylene blue eosinate in methanol; (2) it is not readily soluble during alcohol extraction; (3) in some cells, e.g., Corynebacterium, Mycobacterium, it is removed with ribonuclease pretreatment (although in others, e.g., Spirillum serpens, it is not).

In a few gram negative organisms (e.g., Klebsiella pneumoniae) there is some slight Giemsa staining of the cell wall. These cells also decolorize slowly in the gram staining procedure. Gram positive organisms stained with eosinated methylene blue alone present the same staining picture as gram negative species. Ribonucleaseprotein, as determined by removal with ribonuclease, accounts for most of the staining. However, upon subsequent staining with the Giemsa solution there is an intense red coloration of the cell wall. This staining is prevented with lysozyme digestion for one or two minutes. It is not prevented by either ribonuclease or desoxy-
ribose pretreatment of the cells although ribonuclease does destroy the gram positivity of
the cells.

**DISCUSSION**

Jacobson and Webb (1952), in extensive observations on the specificity of the May-Green-
wald and Giemsa stain for other cells, have shown that desoxyribonucleoprotein only stains red
with Giemsa in their technique; desoxyribonucleic acid, ribonucleoprotein, and ribonucleic acid
stain blue. Therefore, the above results suggest that the substance of E. coli stained by eosinated
methylene blue in methanol is ribonucleoprotein (and ribonucleic acid) and, further, that the affin-
ity of the bacterial nucleus for the Giemsa stain is due to desoxyribonucleoprotein. This comment
on the specificity of the Giemsa stain is directed toward the gram negative bacteria only and, in
particular, toward E. coli.

Giemsa staining without prior exposure to methylene blue eosinate produces dense coloration
throughout the cell with only a faint indication of internal structural differentiation.
Previous staining with methylene blue eosinate in methanol is thus essential for the subsequent
differentiation and localization of the Giemsa mixture. This may not be necessary for all micro-
organisms at all stages of growth. The methylene blue eosinate and Giemsa procedure does not
demand modification during the phases of the growth cycle.

For comparison with this technique, preparations of the same log phase cultures were made
using other nuclear staining methods (figures 9, 10, 11). The nuclear pattern is the same as
that found in uncrushed DeLamater preparations, especially when fixed with cold methanol
(figure 11).

Specific staining of desoxyribonucleoprotein in intact cells (figure 7, a–c) shows the presence
of perinuclear granules analogous to the centrioles of DeLamater. Although these granules are
usually, but not exclusively, found near the cell periphery, they are not “growth points” or
septa.

Removal of ribonucleic acid by ribonuclease is obtained following fixation in cold absolute
methanol or in Chabaud’s fixative; osmium fixation greatly inhibits ribonuclease action. This
may be responsible for the altered appearances noted by several workers (Peters and Wigand,
1953; Murray, 1953).

The use of acid hydrolysis in nuclear staining procedures may be open to criticism; some
shrinkage of osmium fixed cells occurs during acid treatment. However, the nuclear configura-
tions found in (nonhydrolyzed) cells by the eosinated methylene blue-Giemsa method
(figures 6, 7, a–c) resemble closely those described by DeLamater (1953a) for acid hy-
drolyzed cells. In addition, wet mounts observed before dehydration and after staining with
DeLamater’s (1953a) procedure show that the nuclear structures are present and consequently
cannot be the product of the dehydration process. Gross alterations in structure have never been observed following the method out-
lined by DeLamater (1953a) either in hydrolyzed specimens prepared with care or on whole cells
(Methods and Materials).

Mitotic nuclear configurations of E. coli are not due to fat globules pressing on the bacterial
nuclei since the cells contain no free lipid and only a small amount of bound lipid localized in
peripheral granules (Davis et al., 1953).

These facts do not of themselves validate the interpretations of DeLamater (1953a, b, c), for a
mitotic cycle, which he has outlined as an initial basis for further investigation in E. coli. We may
conclude, however, that the chromosomal configurations and divisional stages thereof de-
scribed by DeLamater may be obtained with techniques differing in many important respects.
Hence, they are not merely products of one procedure. Though some modification may be
necessary in the future, results now available are consistent with the cycle he has proposed.

As reported elsewhere (refer to figures 1 and 2 of Mudd, 1953) the nuclei and perinuclear
granules (centrioles), stained red in the intact cell by the eosinated methylene blue and Giemsa
procedure, are distinct from the cytoplasmic organelles (mitochondria) of E. coli procedure.
The ribonucleoprotein is distributed throughout the cytoplasm. The dark and light patterning of
cells stained in Leishman’s alone (figure 3) indicates that the concentration of ribonucleoprotein
in the nucleus must be low in comparison with that of the cytoplasm.

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of the May-Greenwald and Giemsa technique in tissue culture studies.

SUMMARY
The methylene blue eosinate and Giemsa technique of Jacobson and Webb has been adapted for use on certain gram negative bacteria. Specific differential staining of ribonucleoprotein (blue) and deoxyribonucleoprotein (red) has been obtained. Ribonucleoprotein is concentrated largely or exclusively in the cytoplasm where its distribution appears homogeneous and resembles the pattern of densities seen in living cells under phase contrast. Desoxyribonucleoprotein is confined to the cell nucleus and occasional small perinuclear granules.

REFERENCES