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ON THE PRESERVATION OF ANTIGENIC DETERMINANTS DURING FIXATION AND EMBEDDING FOR ELECTRON MICROSCOPY

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The study of the fine structure of cells and cellular bodies by electron microscopy has made great strides forward in the last decade. An important contribution to further development would be specific electron stains with which to identify and localize individual molecules within the cellular matrix. The success achieved by the Coons' fluorescent antibody conjugate technique of staining for light microscopy[1] clearly suggests the use of antibody as the basis for a general and highly specific electron stain. The preparation of two different types of antibody conjugate for electron microscopy has recently been reported.[2, 3]

In order to make effective and general use of an antibody conjugate as an electron stain, however, serious problems of specimen preparation must be overcome. Antibody molecules do not ordinarily penetrate living cells, and cells must therefore be first subjected to suitable treatment. Two general procedures for staining with an immuno-specific electron stain are possible. The cells might first be fixed in a manner appropriate for electron microscopy, which simultaneously renders them permeable to antibody molecules, and then stained with the antibody conjugate, followed by conventional embedding and sectioning of the cells for electron microscopy; or the cells might first be fixed, embedded, and sectioned and then stained. The latter procedure would probably be the most generally useful if it could be developed. The question immediately arises, however, as to whether antigenic determinants in a cell can be subjected to the treatment involved in fixation and embedding and still retain their capacity to bind specific antibody.

Several methods of fixation and embedding for electron microscopy are in use at the present time. In these preliminary studies, our objective was to determine the effect of some of the simpler and more widely used methods on the antigens of several simple systems. To our knowledge, no reports of such studies have yet appeared. The techniques of fixation and embedding ordinarily utilized with the fluorescent antibody conjugate method,[1] which of course do permit the retention of the antibody-binding capacity of many different antigens, are not generally applicable to electron microscopy and are therefore not pertinent in this connection.

The major part of these studies involved the antigens of *Escherichia coli* and *Streptococcus pyogenes*. These organisms were chosen because they are morphologically distinct, and the specificity of staining was therefore easily ascertained in mixtures of the two. By using the fluorescent antibody technique, it was possible

to examine them rapidly and directly with the fluorescence microscope and to determine the effect of fixation and embedding treatments on the antigenic determinants of the two bacteria.

Materials and Methods.—The crystalline proteins bovine pancreatic ribonuclease (RNase) and bovine serum albumin (BSA) were obtained from Armour and Pentex, respectively. The tobacco mosaic virus (TMV) and the cultures of *E. coli* B and of a group-A, Lancefield-type-4 streptococcus were kindly furnished by colleagues at the University of California. Brain heart infusion broth (Difco) and Todd-Hewitt broth (Difco) were used for cultivating *E. coli* and *S. pyogenes*, respectively.

The γ-globulin fractions of rabbit antisera to RNase and BSA were similar to those used in other studies;[4] rabbit antiserum to TMV was part of a preparation by Bradish and Crawford.[5] The γ-globulin fraction of this serum was obtained by precipitation with 40 per cent saturated $(NH_4)_2SO_4$ adjusted to pH 6.8.

For preparation of the streptococcus immune sera, cells from a 48-hour culture were resuspended in 0.85 per cent saline containing 0.4 per cent formalin. Rabbits were given 12 intravenous injections over a three-week period and bled in the fourth week. A similar procedure was used to prepare antisera to formalinized *E. coli*.

Osmic acid fixative at pH 7.3[6] and permanganate fixative[7] were prepared as recommended. Embedding was carried out in a methacrylate monomer mixture by three methods: (1) thermal polymerization at 50° C. for 10 hrs, (2) thermal polymerization at room temperature for 2 days in the presence of an accelerator, or (3) ultraviolet-induced polymerization at −10°C for 10 hrs in the presence of a photosensitizer. The monomer mixture in each case consisted of three parts of butyl methacrylate to one of methyl methacrylate and contained one per cent benzoyl peroxide. In addition, to this mixture was added either 0.5 per cent dimethylaniline for method (2) or 0.2 per cent benzoin for method (3). For the ultraviolet irradiation, a Westinghouse Black Light Blue fluorescent lamp, F15T8/BLB, was used in a cold room at −10°C.

To determine the effects of the fixatives on the soluble antigens RNase, BSA, and TMV, 0.10 ml. of a concentrated protein solution was mixed with 2.0 ml. of fixative at 0°C for 30 min. The solutions were then dialyzed overnight at 4°C against 3 liters of phosphate buffer, pH 7.5, $\Gamma/2$ 0.1. If these solutions contained insoluble matter, they were then centrifuged to remove it. A portion of this solution was then mixed at 37°C with a solution of γ-globulin containing homologous antibody, and the flocculation time and the amount of precipitate formed was compared with a control containing the same amount of untreated antigen and homologous antibody, and with another control containing the treated antigen and normal γ-globulin.

The bacterial cells were fixed and embedded by the following procedure. Equal volumes (300 ml or less) of overnight cultures of the two bacteria were mixed, centrifuged, washed once with 40 ml of phosphate buffered saline (PBS) at pH 7.0, centrifuged again, and resuspended in 5 ml osmic acid fixative. After fixation at 0°C for 10 minutes, the mixture was diluted with 35 ml of cold PBS and the cells were centrifuged down. During fixation, the cells formed into clumps, which proved to be undesirable for purposes of specific staining; these clumps were therefore broken up by resuspending the cells in PBS and drawing them in and out of a syringe fitted with a 20-gauge needle until microscopic examination showed most of the cells to be singly dispersed. The PBS wash was repeated twice using 40 ml volumes at 0° C.

The cells were then washed successively for 10–20 minutes at room temperature once with 15 ml each of 25%, 50%, 75% ethanol, twice with 15 ml of absolute ethanol, once with 5 ml of a 1:1 mixture of methyl methacrylate and absolute ethanol, once with 5 ml of methyl methacrylate, and twice with 5 ml of a 3:1 mixture of butyl and methyl methacrylates. The cells were divided at this point into three portions and resuspended in the respective polymerizing mixtures used for the three polymerization methods discussed above. After transfer to gelatin capsules, they were centrifuged, the polymerizing mixture was replaced, and embedding was carried out.

Fluorescence microscopy was performed with a Zeiss fluorescence microscope illuminated with an Osram HBO 200 mercury vapor burner. The filter system consisted of the UG 2 and UG 5 exciter filters and the GG 4 barrier filter. A monocular microscope equipped with a cardioid darkfield condenser and a 40X oil immersion fluorite objective was used. Fluorescent antibody conjugates were prepared with fluorescein isothiocyanate and used in the direct staining method

described by Coons and Kaplan.[8] Those labeled antibody preparations that stained nonspecifically were first absorbed with the heterologous organisms.

Smears were prepared from cells after each of the various steps of fixation, dehydration, and embedding and were then stained. With the embedded cells, the intense blue autofluorescence of polymerized methacrylate made it necessary to dissolve the polymer away from the cells before staining. This was accomplished by exposing the embedded sections to benzene overnight at 4°C, followed by washes with benzene-ethanol, ethanol, and PBS. Smears of these cells were then made and stained. The intensity of staining was arbitrarily graded in 5 steps from ++++ to −, each step corresponding to about a 25 per cent decrease in intensity of fluorescence. Staining specificity was confirmed by (1) staining mixtures of the two bacteria which could be distinguished morphologically, (2) inhibiting staining with unlabeled antibodies,[9] and (3) removal of labeled antibodies by the addition of homologous bacterial suspensions.

Results.—Treatment of the soluble antigens RNase, BSA, or TMV with osmic acid fixative under the conditions described produced no change of color of the protein solution nor any other evidence of chemical reaction. The flocculation time and the amount of specific precipitation by antibody were unaffected. On the other hand, RNase and BSA solutions treated with $KMnO_4$ fixative acquired a deep brown color but remained soluble, while a similarly treated TMV solution showed a copious brown precipitate. None of these proteins gave any visible precipitate when treated with their homologous antibodies.

Osmic acid fixation of previously dried smears of the bacteria resulted in a high degree of nonspecific staining. If, however, the cells were fixed in suspension instead of in smears and washed in suspension with PBS by the methods described above, the intensity and specificity of staining were the equivalent of those found with ethanol-fixed cells. Osmic acid-fixed cells which were dehydrated, embedded in methacrylate, and treated as described to remove the methacrylate polymer, stained with a +++ intensity compared to ++++ for the cells which were fixed only. This was true for both types of cells. This drop in staining intensity was a gradual one occurring through the course of the procedure rather than at any one step in it. The specificity of staining was found to be maintained throughout the procedure, as tested by all three methods described in the previous section, and is illustrated in Figure 1. There was no observable difference in staining produced by the three different methacrylate embedding methods used.

Discussion.—Several methods of fixation for electron microscopy have been developed. Fixation by rapid freezing, followed by sublimation of the frozen water at very low temperatures[10] or followed by substitution of the frozen water by a polymerizable monomer at very low temperatures,[11] would appear to be the method least likely to inactivate antigenic determinants. However, these are still cumbersome and time-consuming methods, and chemical fixatives are more widely used. We have shown that treatment with osmic acid fixative has no serious effect on the capacity of several different types of antigens to bind specific antibody. Our results suggest further that protein antigens generally will be found to be unaffected by osmic acid, since there was no indication that any chemical reaction had occurred with the several protein antigens examined. It is therefore quite likely that osmic acid fixation will be a generally useful technique in preparing specimens for staining with antibody conjugates for electron microscopy. If other types of antigen systems are encountered which are inactivated by such treatment, resort may be had to rapid freezing methods.

On the other hand, $KMnO_4$ fixative completely inactivated these same protein antigens, which is not too surprising in view of the powerful oxidizing capacity of $KMnO_4$. It is clear therefore that this fixative is not useful for our purposes.

Embedding of cells and tissues for electron microscopy has been carried out successfully and reproducibly in a variety of polymerizing systems. In many of these, the monomers contain reactive chemical groups which can attack groups in the antigenic sites of proteins and other macromolecules. For example, the epoxide groups of the Araldite embedding system[12] are likely to react with amino and other groups of antigenic components[13] and possibly inactivate them in the process.[14] For these preliminary experiments, therefore, we confined ourselves to methacrylate embedding, since in a vinyl polymerization the small concentration of free radicals and their selective reactivity for monomer molecules were expected to favor retention of antigenic activities. It was not possible to stain sections of the embedded cells directly with fluorescent Ab because of the intense autofluorescence of the polymerized methacrylate; instead, the stain was applied after the polymer was dissolved away from the cells. This permitted us to determine whether the cells had retained their antigenic activities during the embedding process, but no information was obtained as to whether cells within the embedded matrix could be stained. We did indeed find that polymethacrylate embedding, even if carried out at 50°C, permitted the retention of at least a substantial fraction of the antibody-binding capacity of the osmic acid-fixed bacteria.

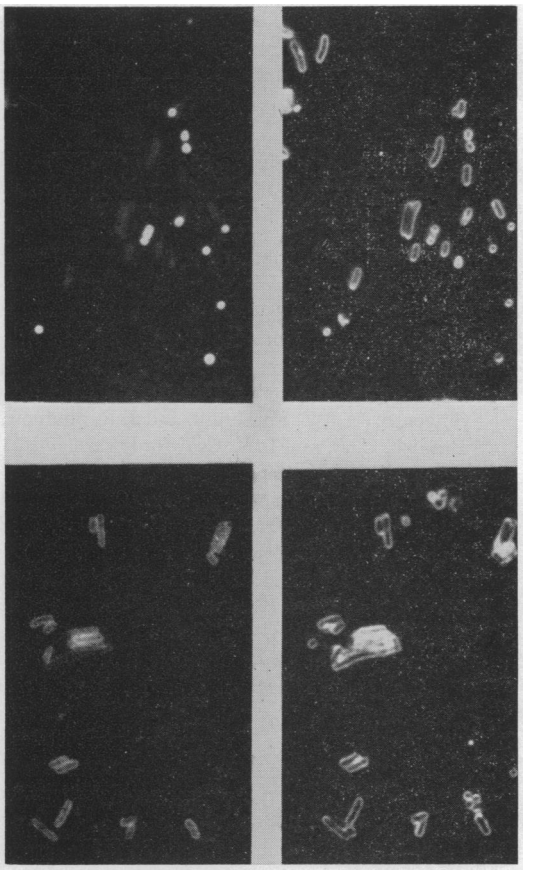

FIG. 1.—Photomicrographs of mixtures of *E. coli* and *S. pyogenes* which were fixed in osmic acid and embedded in methacrylate as described in the text. The two top pictures are of a specimen which was stained with fluorescein-labeled anti-*S. pyogenes* antibodies, and examined in visible light (top right) and in the ultraviolet (top left). The spherical *S. pyogenes* cells were stained specifically. The two bottom pictures are of a specimen similarly prepared, but stained with fluorescein-labeled anti-*E. coli* antibodies, and examined in visible light (bottom right) and in the ultraviolet (bottom left). At least 10 well resolved *S. pyogenes* cells may be observed to be not stained, and the rod-like *E. coli* cells were therefore stained specifically.

These results are too few to permit any generalizations to be made about the preservation of a wide variety of antigenic determinants through osmic acid fixation and methacrylate embedding. This is particularly so because the antigens of

bacteria are many and complex,[15] and it is not evident whether they are more or less stable to inactivation than viral or other types of antigens. Nevertheless, these results do strike an optimistic note for further investigations. Furthermore, these studies have provided us with a model system of antigenically-active and suitably embedded bacterial cells with which to test various antibody conjugates as specific stains for electron microscopy, and such investigations are in progress.

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STUDIES ON THE COMPOSITION OF THE PROTEIN FROM ESCHERICHIA COLI RIBOSOMES

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Much recent evidence indicates that protein synthesis takes place on the ribonucleoprotein particles now commonly known as ribosomes. Both *in-vitro* and *in-vivo* experiments with radioactive amino acids have produced evidence that peptide linkages are formed on ribosomes and that the growing polypeptide chains remain attached to ribosomes until their completion. A question thus poses itself: What fraction of the total protein of the ribosomes consists of growing or newly