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Hydroxybenzene Compounds as Cytoplasmic Fixatives.*

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Because of a renewed interest in cytoplasmic components quickened by the development of methods for centrifugal fractionation of these constituents, this investigation was undertaken in an effort to develop a simple yet effective histological method for the study of the cell cytoplasm and, more particularly, of such structures as the mitochondria. At the present time 3 types of agents[1] are employed in fixation of the cytoplasm; heavy metals, osmium tetroxide, and formaldehyde. As the protein precipitating properties of the heavy metals have been investigated rather extensively, further consideration of these compounds did not appear to be warranted. Osmium tetroxide gives adequate preservation of only a small layer of cells within a tissue block, its "fixing" properties possibly being due to its action as a strong oxidant. The uncontrollable nature of such oxidation, however, severely limits the usefulness of this reaction in the fixation of tissues. Formaldehyde, on the other hand, is a weak reducing agent, and although in itself it does not yield exceptional results, suggests that other reducing compounds might be of value in the preservation of cytoplasmic constituents.

In line with this reasoning, trihydroxybenzene or pyrogallol was considered as it seemed to be well suited for our purposes, being a relatively strong reducing substance, when in alkaline solution, as is required for the maintenance of cytoplasmic detail. Preliminary investigations revealed that weak solutions of pyrogallol preserved the mitochondria but caused excessive alteration of other cell structures. As hydroxybenzene compounds combine with formaldehyde, pyrogallol-formol mixtures were then tried and found to give more desirable results. Investigation of the unsubstituted dihydroxybenzenes in combination with formaldehyde revealed that hydroquinone and catechol give rather poor fixation but that resorcinol approximates pyrogallol in effectiveness.

Pyrogallol-formol Mixtures. As alkaline solutions of pyrogallol and formaldehyde take up oxygen readily and acid solutions form high polymers on standing, all solutions must be made up shortly before use. This is a relatively minor disadvantage, however, since weighed amounts of pyrogallol can be stored in the dry vials in which the tissue is to be fixed, and the desired amount of alkalinized formaldehyde solution added just prior to use.

For the fixation of tissue pieces, up to 5mm in thickness, solutions of 3.5 to 7% pyrogallol dissolved in 4% formaldehyde alkalinized with 0.1 cc of N. NaOH per 10 cc give the best results. Fixation is complete in 6 to 8 hours but periods of fixation as long as 24 hours do not appear to be particularly harmful. When small tissue fragments or thin layers of cells, such as those obtained in tissue culture, are to be fixed, the time of fixation must be reduced, 15 to 30 minutes sufficing in the latter case if the plasma clot has been removed. After fixation, the tissues can be immediately dehydrated in varying strengths of alcohol and imbedded in paraffin or paraffin mixtures. If the tissues are allowed to stand in 70 to 80% alcohol for periods of weeks or months, they darken considerably and harden somewhat, and this has been found to interfere, to some extent, with certain of the staining reactions.

Tissue blocks, prepared as outlined, section easily, and may be cut at thicknesses of

* This research was begun in the laboratories of Pathology and Bacteriology of the Rockefeller Institute for Medical Research.

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1 Baker, J. R., *Cytological Technique*, London, Methuen, 1933, p. 131.

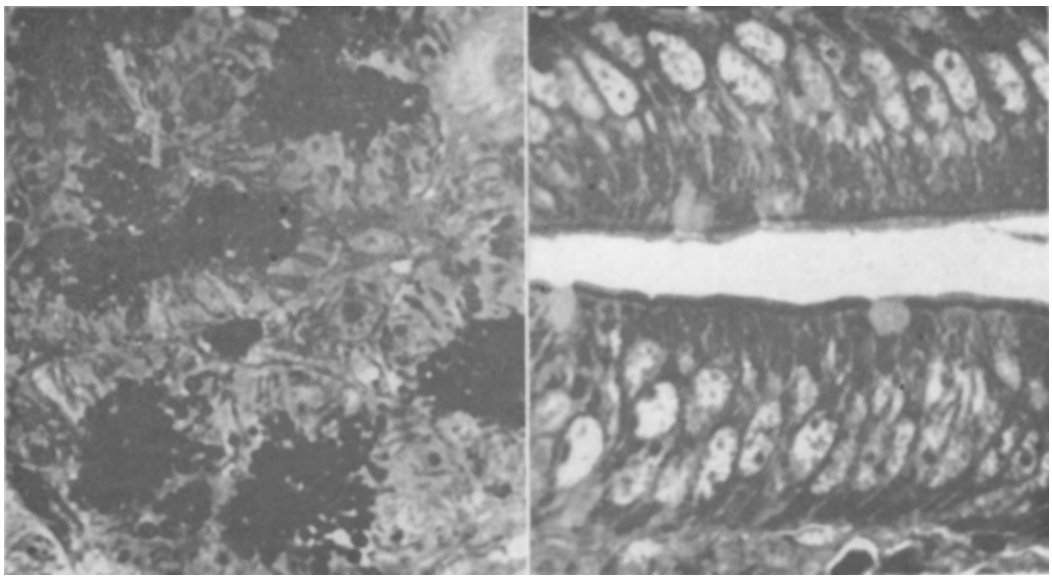

FIG. 1.

A section of pancreas of a non-fasted adult guinea pig showing the centrally located zymogen granules and the more peripherally located elongated mitochondria. Fixed in 9% resorcinol in 4% alkalinized formaldehyde, sectioned at 2 micra, and stained by the Altmann-Bensley procedure. Mag. × 2100.

FIG. 2.

A section of epithelium from the duodenum of a non-fasted mouse showing particularly the mitochondria and the brush border of the cells. Fixed in 7% pyrogallol in 4% alkalinized formaldehyde, sectioned at 2 micra, and stained by the Altmann-Bensley procedure. Mag. × 2100.

Photomicrographs taken by H. W. Morris.

1 to 2 micra without much difficulty. The reaction of various staining procedures following this fixation is interesting. The iron-hematoxylin methods give the best results. Mitochondria and other cytoplasmic granules stain sharply, and the nuclei show good detail as do such structures as basement membranes, terminal bars, and brush borders. The Altmann-Bensley staining procedure also gives good results if the tissue slices are not treated with potassium permanganate and oxalic acid or with potassium dichromate. Schleicher's modification of Mallory-Heidenhain connective tissue stain[2] gives fairly good results although the azocarmine overstains to some extent, but routine hematoxylin and eosin methods must be preceded by treatment with potassium permanganate and oxalic acid to insure staining with the hematoxylin. After the tissues have been stored in alcohol for some time and have darkened considerably, the results obtained with all of the above staining procedures, except iron-hematoxylin, are less satisfactory.

Resorcinol-formol Mixtures. The procedure for the preparation of resorcinol-formol mixtures is the same as that for the pyrogallol-formol solutions already outlined. Tissue pieces are well fixed in solutions of 4.5 to 9% resorcinol in 4% alkalinized formaldehyde. The time necessary for fixation and the subsequent treatment of the tissue blocks are the same as for the pyrogallol-formol mixtures, but tissue fixed with resorcinol-formol darkens much more slowly when stored in alcohol so that storage may be somewhat longer without altering the staining reactions.

Tissue prepared in this manner is somewhat softer and sections with greater ease than when fixed with pyrogallol-formol solu-

[2] Schleicher, E. M., *Am. J. Clin. Path.* (Tech. Supl.), 1943, **7**, 35.

tions. Their reaction to various staining procedures is, however, rather different. The Altmann-Bensley method gives the best results for mitochondria. The tissue slices should not be treated with potassium permanganate and oxalic acid, and, although good results are obtained if potassium dichromate is employed, somewhat clearer staining is produced by omitting this step. Routine hematoxylin and eosin methods, Schleicher's modification of Mallory-Heidenhain connective tissue stain, and Pappenheim's methyl green-pyronin method give good results following resorcinol-formol fixation. However, although the nuclei and cytoplasmic ground substance stain well with iron-hematoxylin, the mitochondria do not show up distinctly even after the tissue has been allowed to stand in alcohol until it has darkened.

Discussion. Some investigation of the action of these hydroxybenzene-formol mixtures has been carried out, and the results warrant terse mention here. Although the pH of any fixative is of utmost importance where the cytoplasm is concerned, this aspect is easily adjusted in the solutions outlined above, for, with the addition of NaOH, the weakly acidic pyrogallol and resorcinol form buffer systems. The point of greatest buffering power of these systems is in the neighborhood of pH 9, but with the relatively high concentrations used for fixation, 7% pyrogallol for instance, increasing the concentration of NaOH from .01 N to .02 N only increases the pH from 7.4 to 7.7 and the latter is not excessive for tissue fixation.

The effect of these solutions upon cytoplasmic extracts is also of some interest. Although neutralized formaldehyde does not cause precipitation of the proteins in such extracts and even protects them from subsequent precipitation with alcohol in concentrations of 50% or less, the hydroxybenzene-formol mixtures cause an immediate and heavy precipitation of the protein components. When purified preparations of mitochondria are so treated, the particles agglutinate, but microscopic examination reveals that the individual elements are not appreciably altered morphologically. That they have been chemically modified, however, is evidenced by the fact that after treatment they are insoluble in NaOH even in concentrations of 0.5 N whereas untreated mitochondria dissolve readily in solutions of NaOH that exceed 0.05 N.

The reaction of cells in tissue culture was studied by the method of dark-ground illumination as given by Strangeways and Canti.[3] As the fixing solutions were drawn across the cells, the cell boundaries became sharply demarcated and, although a few of the finer processes were seen to retract, in general the original outlines of the cells were well maintained. The ground substance of the cells became much more brilliantly illuminated, but no discrete particles of precipitated protein nor obvious precipitation patterns appeared. The perinuclear refractile bodies, which probably represent the Golgi apparatus,[4] showed little or no tendency to fuse, and apparently were essentially unaltered as they dissolved completely when 95% alcohol was drawn through the chamber. The mitochondria became less noticeable because of the increased density of the illumination of the ground substance, but careful inspection revealed no evidence of change in their size or shape either with fixation or with subsequent treatment with alcohol and ether. No alteration of size or shape of the nuclei or nucleoli was noted, although the same general increase in density of illumination that was observed in the cytoplasm was also seen in these structures. In general, then, direct observations of tissue culture cells during fixation and subsequent treatment with alcohol and ether substantiated the impression gained from a study of sectioned material that these fixatives distort the cell very little and preserve the mitochondria very well.

The results obtained with these fixing solutions have been especially satisfactory because, in addition to the preservation of cytoplasmic detail, such structures as brush borders, terminal bars, elastic membranes in the arterial system, and basement membranes

[3] Strangeways, T. S. P., and Canti, R. G., *Quart. J. Micro. Sci.*, 1927, **71**, 1.

[4] Porter, K. R., Claude, A., and Fullam, E. F., *J. Exp. Med.*, 1945, **81**, 233.

have been well shown in such preparations. Red cells, however, appear badly distorted in the larger vessels although they are rather well preserved in the capillaries and arterioles. This deleterious effect is probably not of osmotic origin as it occurs when iso-osmotic solutions of hydroxybenzene-formol are used as well as when .85% NaCl is added to such solutions. Another undesirable feature of these fixatives is that when tissue blocks remain in them sufficiently long to allow penetration and fixation throughout the block, the cells near the surface are over fixed. This over fixed zone is, however, not very thick when the tissue has been fixed 6 to 8 hours and does not, as a rule, interfere with the study of the tissue.

Summary. Methods for the fixation of the cell cytoplasm employing pyrogallol or resorcinol in neutralized formaldehyde solution are outlined. Tissue pieces fixed with these solutions have, in general, shown few signs of distortion, and the protein cytoplasmic elements of the cells have appeared well preserved, such structures as mitochondria, secretion granules, and some of the specific types of granulation being well maintained. The advantages of these methods lie in the simplicity and rapidity of the procedures, the ease with which the tissue can be sectioned at a thickness of 1 to 2 micra, and the generally satisfactory results obtained.

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Antipyridoxine Activity of 2, 4-Dimethyl-3-Hydroxy-5-Hydroxymethyl-pyridine in the Chick.

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In the course of investigations on the vitamin activity of pyridoxine analogs, it was discovered that 2,4-dimethyl-3-hydroxy-5-hydroxymethylpyridine had very strong antipyridoxine properties. Growth and survival of pyridoxine-deficient chicks in curative assays were used as the criteria of response. This substance, a desoxypyridoxine, had been studied in pyridoxine-deficient rats by Möller[1] and associates and by Unna,[2] both of whom found no pyridoxine activity. No indication of antipyridoxine activity was reported by these investigators.

Day-old female single comb White Leghorn chicks were fed a commercial starting ration for 3 days and then were given a purified diet (Table I) deficient in pyridoxine. After 6 days on this deficient diet, when many chicks were beginning to lose weight, the chicks were divided into groups of 7 selected in such a way that all groups were as nearly alike as possible in weights of the individual chicks. The test substance was given in single oral doses to each chick on the 11th day of age and on the following 3 alternate days. Three or more dose levels of pyridoxine in the range from 0 to 24 μg per dose were given in each assay to establish the standard curve of response for that assay. New standard solutions of pyridoxine and of desoxypyridoxine were prepared in water for each assay and kept under toluene in a refrigerator for the 6-day dosing period. The chicks were weighed before each dose and on the second day after the last dose was given. The pyridoxine-deficient diet was fed to all groups during the assay period.

Two 100 μg doses of desoxypyridoxine were fatal to pyridoxine-deficient chicks even when each dose was preceded by a 16 μg dose of pyridoxine (Table II). Furthermore, a level of the analog as low as 16 μg per

[1] Möller, E. F., Zima, O., Jung, F., and Moll, Th., *Naturwiss.*, 1939, **27**, 228.

[2] Unna, K., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 122.