Cytochemical Differentiation between the Pentose and Desoxypentose Nucleic Acids in Tissue Sections

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With Plate 7

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ANIMAL tissues contain two types of nucleic acid: the desoxypentose, or thymo-, nucleic acids whose distribution is confined to the nucleus; and the pentose, or yeast, nucleic acids which can occur in both nucleus and cytoplasm. For many years it has been possible to identify desoxypentose nucleic acid by means of the Feulgen reaction, although, as has been frequently pointed out (Lison, 1936; Stowell, 1943), this must be carried out under carefully standardized and controlled conditions if its results are to be reliable. No corresponding test for pentose nucleic acids existed until Brachet (1940, 1941) showed that it was possible to identify pentose nucleic acids by comparing sections stained with basic dyes with other sections similarly stained following treatment with a pancreatic extract containing the enzyme ribonuclease. Therefore a distinction between the two types of nucleic acid may be made by applying both the Feulgen and Brachet tests to the same material. However, since the stains used in the two cases are different, and one set of sections is subjected to acid hydrolysis and the other is not, it may be difficult to relate the two cytological appearances to one another.

The present paper describes a simple differential test for the two types of nucleic acid involving the use of highly purified enzyme preparations. It differs from Brachet's (1940) test in two particulars: (1) all sections are stained with the same set of dyes after incubation in ribonuclease, desoxyribonuclease, or plain buffer solution; so that comparisons between the differently

treated sections are more easily made; (2) an attempt has been made to make the test more reliable by the use of highly purified enzymes, with precautions to restrict their action to a single substrate.

The test itself is performed as follows: Tissues are fixed by perfusion with Carnoy, Zenker, or chilled acetone,[1] dehydrated, and embedded by the usual methods. After Bouin or formalin fixation the method gives less clear-cut results. Blocks are cut at 10μ, and adjacent sections attached to each of four slides, which are dewaxed in choloroform, rehydrated, and washed in three changes of distilled water. One slide is then incubated for 1 hour at 56° C. in M/15 acetate-veronal buffer of pH 6·75 containing 1 mg. per 100 c.c. of crystalline ribonuclease, prepared according to Kunitz (1940). Since it has been suggested that even crystalline ribonuclease has some residual proteolytic activity (Cohen, 1945), due presumably to adsorbed impurity, this is destroyed by heating the enzyme solution to 80° C. for 10 minutes before use. A second slide is incubated for 1 hour at 37° C. in an M/40 acetate-veronal buffer of pH 7·5 containing 1 mg. per 100 c.c. highly purified pancreatic desoxyribonuclease, prepared as described by McCarty (1946). Since this enzyme is heat-labile, the solution was not heated to remove any possible residual proteolytic activity. It was found, however, as also noted by McCarty (1946), that at the dilution used proteolysis is negligible during one hour's incubation. The enzyme solution also contained gelatine at a concentration of 0·01 per cent. as protective colloid, and also 0·003M $MgSO_4$, the magnesium ions being necessary to activate the enzyme. The remaining two slides are used as controls, one being incubated in each of the buffers made up exactly as described above, only without the added enzyme. After treatment the four slides are washed in several changes of distilled water and then passed together through the following two solutions: (1) 0·50 per cent. celestine blue in acid solution as described by Lendrum (1935); duration

[1] Acetone fixation was investigated as it allows the performance, upon adjacent sections, of the reactions for alkaline and acid phosphatase described by Gomori (1941) and Wolf, Kabat, and Newman (1943), whereby nucleic acid distribution and phosphatase activity may be correlated.

of staining—5–15 minutes followed by 10 minutes washing in running tap-water; (2) 0·25 per cent. Pyronin G (pyronin 0·25 gm.; 96 per cent. alcohol 2·5 c.c.; glycerin 20 c.c.; phenol 0·5 gm.; water to make 100 c.c.); duration of staining 30 minutes. Following this the slides are rinsed in tap-water, blotted dry with filter paper, and transferred direct to a mixture of three volumes of xylene with one of absolute alcohol. This mixture, originally suggested by Unna, dehydrates the sections without extracting much of the pyronin; celestine blue is not extracted by alcohol. Finally, the slides are cleared in xylene and mounted in neutral Canada balsam.

On slides so treated the differential distribution of desoxypentose and pentose nucleic acids may be ascertained by comparing the two enzyme-treated slides with the two controls. For example, fig. 1, Pl. 7, is a photomicrograph of a control section of the cerebellum of a guinea-pig, fixed in acetone and stained in celestine blue–pyronin. The nuclei of the granular layer stain intensely with celestine blue, while the Purkinje cell layer is strongly coloured by pyronin. Fig. 2, Pl. 7, shows the section treated with desoxyribonuclease. The Purkinje cells still have a strong affinity for pyronin, while the nuclei of the granular layer are hardly stained, and then by pyronin rather than celestine blue. In fig. 3, Pl. 7, the section treated with ribonuclease is shown. The nuclei of the granular layer take the celestine blue strongly, while the Purkinje cells are pale and hard to differentiate from the background. From this we may conclude that the Purkinje cells are rich in pentose nucleic acid, but relatively poor in desoxypentose nucleic acid, while there is a considerable concentration of the latter in the nuclei of the granular layer immediately below them. Figs. 4, 5, and 6, Pl. 7, show that the test may be used to differentiate the two acids within the cells themselves. Fig. 4, Pl. 7, shows a Purkinje cell of a type very common in the cerebellum. The nucleolus stains strongly with pyronin, while there is a dense cap of pyronin-stained substance on the nuclear membrane and a diffuse granular zone of pyronin staining in the cytoplasm. The nucleus also contains matter staining with celestine blue. In fig. 5, Pl. 7, a similar cell is shown, this time after

treatment with desoxyribonuclease. The dense pyronin-stained nucleolus, nuclear membrane, and cytoplasm remain, but the blue staining masses in the nucleus are absent. A third cell, stained after incubation with ribonuclease is shown in fig. 6, Pl. 7. Here the cytoplasmic granules and the cap on the nuclear membrane staining with pyronin have been removed, but the nucleolus is still visible staining faintly with celestine blue rather than pyronin, as do the other masses within the nucleus. From this test it may be concluded that cells of this type contain both types of nucleic acid, distributed in the following way: (1) pentose nucleic acid; there is a large amount of this acid in the cytoplasm, in addition to especial concentrations at the nuclear membrane and in the nucleolus; (2) desoxypentose nucleic acid; this is confined to the nucleus, there being a small amount in the nucleolus, while the rest is present elsewhere within the nucleus.

Tests with enzymes of the type described above have recently been severely criticized by Danielli (1946) on two main grounds: firstly, that enzyme preparations, however highly purified, are rarely specific for one substrate only; secondly, that removal of part of a cell by nucleases does not prove this part of the cell to be composed mainly of nucleic acid, since there may be only a small amount of nucleic acid present in the key position of a matrix binding two masses of protein material together; also nucleic acids within the cell may be protected from the action of enzymes by a shell of protein. In the above test an attempt has been made to guard against the first of these objections by precautions designed to ensure the specificity of the enzymes used. The important point is that while both enzymes may have slight proteolytic activity, which can be rendered ineffective by heating in the one case and dilution in the other, each enzyme only attacks one of the two types of nucleic acid, and has been shown to be entirely without effect upon the other (Kunitz, 1940; McCarty, 1946). Figs. 7 and 8, Pl. 7, show the specificity of the McCarty enzyme. Fig. 7 shows the granular layer of the guinea-pig cerebellum, stained by the Feulgen technique. Fig. 8 shows an adjacent section, stained in Feulgen after incubation in buffer and desoxyribonuclease. As regards the second objection,

it must be remembered that the above test is performed upon sections of fixed tissues. Under these circumstances it must be expected that the proteins have been made insoluble. Removal of nucleic acid acting as a matrix between protein masses need not therefore cause one protein moiety to go into solution. It is far more likely that the nucleic acid itself will be removed and the protein left as part of the section attached to the slide. Moreover, it is unlikely that protective protein shells, particularly as monolayers, will exist in fixed tissues. Did they exist, they would prevent staining by dyes as much as enzyme action.

It must be borne in mind, however, that difficulty may be experienced in relating the distribution of nucleic acids as revealed by the above test to the living cell. This is, however, a difficulty which attends the interpretation of all images seen in fixed and stained sections. In addition, it must be emphasized that a negative reaction to the above test does not indicate that either type of nucleic acid is absent from the section investigated; the substance may be present, but in such a physical or chemical state that it does not react with the enzyme used. What the test does is to enable us to distinguish the two types of nucleic acid when they are present. With these reservations, it is claimed that the above test provides a simple means of differentiating pentose from desoxypentose nucleic acid in sections of fixed tissue.

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Addendum. Since this paper went to press, Catcheside and Holmes (personal communication) have reported using McCarty's (1946) pancreatic desoxyribonuclease to remove desoxypentose nucleic acid from chromosomes. They also found the enzyme to be almost without proteolytic action.

Explanation of Plate

All figures are untouched photomicrographs of sections stained, except where otherwise stated, by the celestine blue–pyronin technique.

Fig. 1.—Longitudinal section through a convolution of the cerebellum of the guinea-pig, after 1 hour's incubation in M/40 acetate-veronal buffer at pH 7·5. Note: granular layer of nuclei; Purkinje cell layer; white matter.

Fig. 2.—Adjacent section to fig. 1, after 1 hour's incubation in M/40 acetate-veronal buffer pH 7·5 containing 1 mg. per 100 c.c. highly purified desoxyribonuclease.

Fig. 3.—Adjacent section to fig. 1, after 1 hour's incubation in M/15 acetate-veronal buffer pH 6·75 containing 1 mg. crystalline ribonuclease per 100 c.c.

Fig. 4.—Single Purkinje cell from the cerebellum of the guinea-pig after 1 hour's incubation in M/15 acetate-veronal buffer pH 6·75.

Fig. 5.—Single Purkinje cell from the cerebellum of the guinea-pig after 1 hour's incubation in M/40 acetate-veronal buffer pH 7·5, containing 1 mg. per 100 c.c. highly purified desoxyribonuclease.

Fig. 6.—Single Purkinje cell from the cerebellum of the guinea-pig after 1 hour's incubation in M/15 acetate-veronal buffer pH 6·75, containing 1 mg. per 100 c.c. crystalline ribonuclease.

Fig. 7.—Transverse section of a convolution of the cerebellum of the rabbit, after 1 hour's incubation in M/40 acetate-veronal buffer pH 7·5. Feulgen reaction.

Fig. 8.—Adjacent section to fig. 7, after 1 hour's incubation in M/40 acetate-veronal buffer pH 7·5, containing 1 mgm. per 100 c.c. highly purified desoxyribonuclease. Feulgen reaction.

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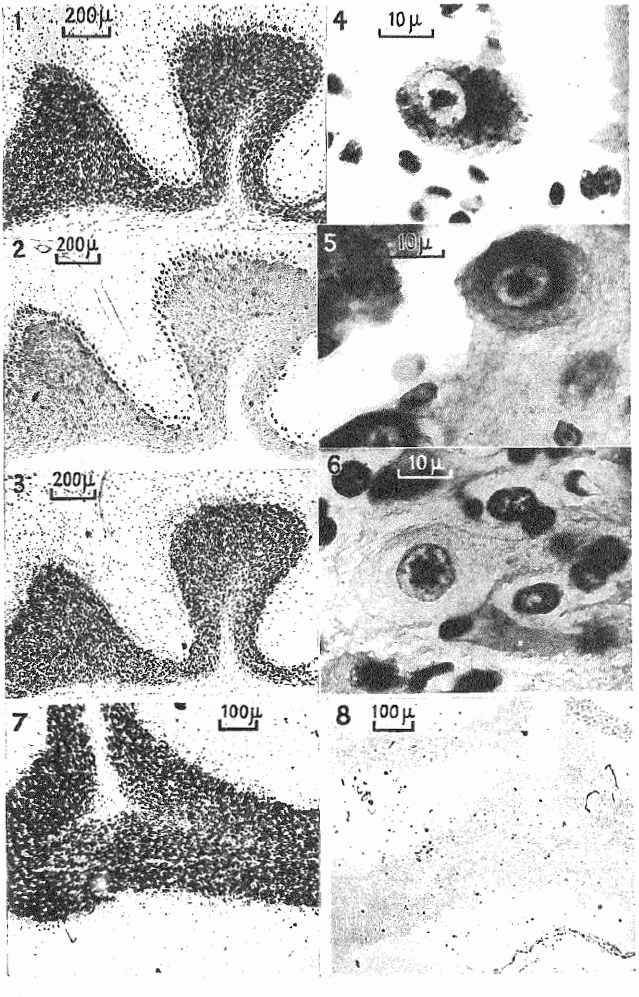

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