The Histochemical Recognition of Phenols, especially Tyrosine

By JOHN R. BAKER

(*From the Cytological Laboratory, Department of Zoology, University Museum, Oxford*)

SUMMARY

Sections of tissue embedded in collodion are heated in an acidified aqueous solution of mercuric sulphate and sodium nitrite. A red colour denotes the presence of phenols (generally the tyrosine of proteins). The reagent is more rational than Millon's and most of the modifications of his method used up till now in histochemistry. It gives a particularly strong coloration.

THE purpose of the work described here was to find a test for phenols that would be more sensitive, dependable, and rational than Millon's (1849) and the various modifications of his method that have already been introduced into histochemistry. Of these, the test of Bensley and Gersh (1933) is probably the most often used; but it was used by them for frozen-dried tissues, and it generally gives a feeble colour with sections of fixed material.

It was shown by Meyer (1864) that a red colour is produced when tyrosine is treated with mercuric chloride in the presence of potassium nitrite in acid solution. Millon's test depends in reality on a similar reaction, nitrite being formed in the preparation of the reagent. The various authors who have introduced tests resembling Meyer's to replace Millon's have all used sodium or potasium nitrite, but they have worked with different salts of mercury. Lintner (1900) used mercuric nitrate, Nasse (1901) the acetate, and Folin and Ciocalteu (1927) the sulphate. The very sensitive test devised by the latter authors was perfected and used quantitatively by Folin and Marenzi (1929).

Mirsky and Pollister (1946) used mercuric sulphate, sulphuric acid, and sodium nitrite as a test for tyrosine in their study of 'chromosin'. They found that their reagent dissolved histone from their material, which had not undergone previous fixation. To prevent this, Pollister and Ris (1947) used trichloracetic acid instead of sulphuric.

It is claimed that in all these tests the reaction proceeds in two stages (Vaubel, 1900; Gibbs, 1927). A nitrosophenol is formed first, by the substitution of -NO for -H in a position *ortho-* or *meta-* to the hydroxyl of the phenol. The red compound is then formed, apparently by the inclusion of mercury in a new ring that also includes the nitrogen of the nitroso-group. Nearly all phenols, except those that are doubly substituted in the *ortho* or *meta* positions, react positively, though thymol provides a partial exception. Tyrosine is naturally positive.

I applied both Nasse's and Folin's reagents to sections of fixed tissues, and obtained a much stronger reaction with the latter's. I then set out to perfect a histochemical test for phenols, based on Folin's method.

[Quarterly Journal of Microscopical Science, Vol. 97, part 2, pp. 161-164, June 1956.]

Experiment showed that particularly strong coloration was given after fixation by formaldehyde, but other common fixatives may be used: Zenker, for instance, or Heidenhain's 'Susa', or Brasil. Susa commends itself because it contains trichloracetic acid, but the colour is not so intense as when formaldehyde is used alone. Helly is unsuitable, because it produces pieces of tissue that react negatively in their outer parts. Even if the fixative used contained mercuric chloride, the section should not be treated with iodine solution.

The concentrations of mercuric sulphate, sulphuric acid, and sodium nitrite that were necessary to give strong coloration with sections of fixed tissue were discovered by empirical experiment.

When a section was heated in the reagent, it generally began to become coloured at about 50° C. Intensification of the colour was especially rapid above 90° C. Excellent results as regards colour were obtained if sections were boiled in the reagent, but this is likely to cause damage with frozen or paraffin sections. It was found, however, that when tissues were embedded in collodion, they could be subjected to quite violent treatment without being injured. The exact nature of the collodion used is of no importance. Low-viscosity nitrocellulose works well. It is strange that collodion sections have so seldom been used in histochemistry.

The colour produced by the reaction is permanent in some mounting media, but fades gradually in Farrants' medium, Canada balsam, and those glycerine-jellies that are neutral or nearly so in reaction. There is no fading with Kaiser's (1880) glycerine jelly (pH 5·5). The phenol in this medium in no way disturbs the result of the test.

These solutions are required:

Formaldehyde-saline. The following solution is suitable:—

Formalin	10 ml
Distilled water	83 ml
Sodium chloride, 10% aq. . .	7 ml

Chalk or marble may be kept in the solution.

Mercuric sulphate solution. Add 10 ml of concentrated sulphuric acid to 90 ml of distilled water. Add 10 g of mercuric sulphate. Heat till dissolved. Cool. Make up to 200 ml with distilled water. The solution is stable.

Sodium nitrite solution, 0·25% aqueous.

(1) Fix the piece of tissue overnight in formaldehyde-saline.

(2) Embed in collodion.

(3) Cut sections at 20–30 μ (or less, for special purposes). Sections may be stored indefinitely in 70 or 80% alcohol.

(4) Bring a section through 50% alcohol to distilled water.

(5) Put 5 ml of the mercuric sulphate solution in a small (50 ml) beaker. Add 0·5 ml of the sodium nitrite solution.

(6) Put the section in the beaker and heat it gently over asbestos gauze until the fluid just boils.

(7) At once remove the beaker from the gauze and allow the fluid to cool somewhat for 2 or 3 minutes.

(8) Wash the section in three lots (about 50 ml each) of distilled water. (Tap-water is unsuitable.) Leave the section at least 2 minutes in each wash. (Long periods are not harmful, as the coloured reaction-product is insoluble.)

The section may now be mounted in any one of several ways. Pure glycerine is a very suitable medium. To mount in Kaiser's glycerine-jelly, pass through 50% glycerine. One can easily bring a section right through the test and mount in glycerine-jelly in 20 minutes. Alternatively one may bring the section up through the alcohols to 95% and then mount in euparal, which dissolves the collodion (see Sheppard, 1921); or one may pass from 95% alcohol through creosote or origanum oil into DPX (Kirkpatrick and Lendrum, 1939).

In the finished preparation phenols are recognized by the red, pink, or yellowish-red colour-reaction. In the tissues of animals the reaction will be due in the great majority of cases to the presence of tyrosine as a constituent of protein.

An excellent object on which to learn the technique is the duodenum of the rabbit, cut out in such a way as to include a part of the pancreas. The part of the duodenum near the opening of the pancreatic duct is the most suitable. The very strong positive reaction in the zymogen granules of the pancreas and in the secretion-granules of certain cells of Brunner's glands is at once noticeable. These cells are the ones called 'dark' by Tschassownikov (1928), on account of the strong staining of the basal region of the cell by iron haematoxylin. The resemblance of these cells to the exocrine cells of the pancreas was remarked by Tschassownikov, and the reaction to this test for phenols provides additional evidence of similarity. These cells, which are especially abundant in the rabbit and hare, appear to deserve further study. The ground cytoplasm and nuclei of the various cells in the section are less strongly coloured than these secretion-granules.

Collagen is almost or quite colourless, because the amount of tyrosine in this protein is so very small. A striking pair of preparations may be made from any organ (such as the mammalian vas deferens) which has a thick layer of collagen fibres lying between components that react positively to the test for phenols. If one section of such an organ be stained by a method for collagen and the other by the test for phenols, the one gives almost a negative image of the other.

I thank Miss B. M. Jordan for practical help.

REFERENCES

BENSLEY, R. R., and GERSH, I., 1933. Anat. Rec., **57**, 217.
FOLIN, O., and CIOCALTEU, V., 1927. J. biol. Chem., **73**, 627.
—— and MARENZI, A. D., 1929. Ibid., **83**, 89.

GIBBS, H. D., 1927. Ibid., **71,** 445.
GILSON, C., 1906. Cellule, **23,** 425.
KAISER, E., 1880. Bot. Centralbl., **1,** 25.
KIRKPATRICK, J., and LENDRUM, A. C., 1939. J. Path. Bact., **49,** 592.
LINTNER, C. J., 1900. Zeit. angew. Chem. (no vol. number), 707.
MEYER, L., 1864. Ann. Chem., **132,** 156.
MILLON, E., 1849. C.R. Acad. Sci., **28,** 40.
MIRSKY, A. E., and POLLISTER, A. W., 1946. J. gen. Physiol., **30,** 117.
NASSE, O., 1901. Arch. ges. Physiol., **83,** 361.
POLLISTER, A. W., and RIS, H., 1947. Cold Spring Harb. Symp. Quant. Biol., **12,** 147.
SHEPPARD, E. J., 1921. J. roy. micr. Soc. (no vol. number), 20.
TSCHASSOWNIKOV, N., 1928. Anat. Anz., **65,** 28.
VAUBEL, W., 1900. Zeit. angew. Chem. (no vol. number), 1125.