Semipermeable Membranes for Improving the Histochemical Demonstration of Enzyme Activities in Tissue Sections*

II. Nonspecific Esterase and β-Glucuronidase

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Summary. Improved histochemical techniques for the demonstration of nonspecific esterase and β-glucuronidase activities in tissue sections are described. With these techniques a semipermeable membrane is interposed between the incubating solution and the tissue sections preventing diffusion of enzymes into the medium during incubation. Fixation of the tissue sections in order to minimize enzyme diffusion but which also causes partial inactivation of the enzymes is no longer necessary. In the histochemical systems the enzymes catalyze the hydrolyzes of 1-naphthol acetate and naphthol AS-BI β-D-glucuronide respectively. The localization of the enzymes is visualized by means of simultaneous coupling of the released naphthol with hexazotized pararosanilin. Problems involved in the histochemical demonstration of the enzymes are discussed.

Introduction

For the development of enzyme histochemical staining techniques, so far attention has been paid exclusively to good preservation of the morphological structure of the sections and an accurate localization of the staining substances formed by the enzyme during the incubation period. For this reason by far most of the enzyme histochemical methods are suitable for morphological studies. For the sake of more precise localization frequently a fixation procedure is applied. This procedure may sacrifice considerable enzyme activity. Moreover those cytoplasmatic enzymes which are not structurally bound may diffuse into the fixative-containing fluid or during the incubating period into the incubating medium (Fahimi and Amarasingham, 1964; Kalina and Gahan, 1965; Altmann and Chayen, 1966; Arnold et al., 1968; Meijer, 1972). The application of macromolecular substances in the incubating media, such as gelatine, dextran, polyvinylpyrrolidone and agar only partially prevents the leaking (Ritter et al., 1971; Meijer, 1972). On the other hand activities of the enzymes by these macromolecular substances can be inhibited (Dahl and From, 1971; Lisý et al., 1971). In a previous publication a method for the histochemical demonstration of acid phosphatase by using a semipermeable membrane interposed between the incubating solution and the tissue sections is described. In this way diffusion of acid phosphatase owing to the relatively high molecular weight of the enzyme is prevented. On the other hand substrate molecules and other components necessary for the staining reaction which have relatively low molecular weights may diffuse through the semipermeable membranes.

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In the present paper semipermeable membrane techniques are described for
the demonstration of activities of nonspecific esterase (EC 3.1.1.1: carboxylie-
ester hydrolase and EC 3.1.1.2: aryl-ester hydrolase) and of \( \beta \)-glucuronidase
(EC 3.2.1.31) in tissue sections. Staining is performed by the simultaneous
coupling of hexazotized pararosanilin with the released naphthol. Fixation of the
sections which causes partial inactivation of both enzymes is for these new
techniques not necessary.

**Material and Methods**

For the histochemical and biochemical experiments tissue specimens of mice, rats and
rabbits were used. The animals were killed by a blow on the head. Small blocks of tissue
specimens were rapidly frozen by immersing in isopentane cooled to \(-150^\circ\) C with liquid
nitrogen. Sections at a thickness of \( 6 \mu \) were cut on a cryostat-microtome. The following
reagents were used in the experiments: 1-Naphthol acetate, naphthol AS-D acetate, naphthol
AS-BI-\( \beta \)-D glucuronide, tetrazonized O-dianisidine and diazonized 4-amino-2,5-dietoxy
benzanilide were obtained from Sigma (St. Louis, U.S.A.). Dextran products with an average
molecular weight of 18000, 40000, 70000, and 150000 were obtained from Poviet and Co.
(Amsterdam, The Netherlands). The polyvinylpyrrolidone products with an average molecular
weight of 40000 and 160000 were obtained from Fluka (Buchs, Switzerland). Gelatine was
obtained from B. D. H. (Poole, Great Britain). Agar (special agar-noble) was obtained from
Difco (Detroit, U.S.A.). Semipermeable cellulose dialysis membranes with an average pore
radius of \( 24 \AA \) (supplier specification) were obtained from Viking Co. (Chicago, U.S.A.). All
other chemicals used were of reagent grade. The mountant cristalite was obtained from
E. Gurr (London, Great Britain).

**Recommended Method**

The preparation of semipermeable membranes, the preparation of incubating vessels and
the mounting of sections on semipermeable membranes are described in the previous communi-
cation.

**Preparation of Incubating-Media in the Vessels**

I. **Nonspecific Esterase.**
   (A) Buffersolution: 9.7 g sodium acetate \( 3 \) \( \text{H}_2\text{O} \), and 14.7 g
   sodium barbiturate is dissolved in 500 ml of distilled water (B) Substrate-solution: 10 mg
   1-naphthol acetate is dissolved in 0.5 ml acetone and diluted with 0.5 ml aqua dest. (C).Pararos-
   sanilin-solution: 400 mg of pararosanilin is dissolved in 10 ml of 2 N HCl. (D) Sodium nitrite-
solution: 400 mg of sodium nitrite is dissolved in 10 ml of distilled water. (E) Agar-solution:
   A \( 2^1/4 \) per cent solution of agar in distilled water is prepared by gently warming on a water-
bath. Incubating-medium: 0.8 ml each of pararosanilin solution and sodium nitrite solution
   is mixed. About 1 min later 5 ml of buffer-solution and 1 ml of substrate-solution is poured
   into the hexazonium pararosanilin-solution and mixed. The acid hexazotized pararosanilin-
solution is neutralized by the alkaline buffer-solution. The pH of the incubation medium is
   adjusted to 5.5. This mixture is poured gradually and mixed thoroughly into 12 ml of warm
   agar-solution. Next 3 ml of the warm incubating medium is poured into each of the vessels
   and cooled to room temperature, after which the solution solidifies. The incubating vessels are
   made ready for use.

II. \( \beta \)-**Glucuronidase.**
   (A) Buffer-solution: 0.2 M acetate-acetic acid solution, pH 5.0.
   (B) Substrate-solution: 3 mg naphthol AS-BI glucuronide is dissolved in 0.3 ml N,N-di-
methyl-formamide. (C) Pararosanilin-solution: 400 mg of pararosanilin is dissolved in 10 ml
   of 2 N HCl. (D) Sodium nitrite-solution: 400 mg of sodium nitrite is dissolved in 10 ml of
distilled water. (E) Agar-solution: A \( 2^1/4 \) per cent solution of agar in distilled water is prepared
   by gently warming on a water-bath. Incubating-media: 0.3 ml each of pararosanilin solution
   and sodium nitrite solution is mixed. About 1 min later 9 ml of buffer-solution and 0.3 ml of
   substrate-solution is poured into the hexazonium pararosanilin-solution and mixed. The pH of
   the incubating-medium is adjusted to 5.0. This mixture is poured gradually and mixed thor-