The Use of Hexazonium-p-Rosanilin in the Histochemical Demonstration of Peptidases*

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Summary. The suitability of hexazonium-p-rosanilin (HP) in the histochemical demonstration of peptidases was investigated. The detection was carried out in cold microtome sections adherent to slides or semipermeable membranes. Alanyl-1-naphthylamide, alanyl-2-naphthylamide, leucyl-2-naphthylamide, leucyl-4-methoxy-2-naphthylamide (all substrates in concentration of 0.4 mg/1 ml of citrate phosphate buffer pH 6.5), y-L-glutamyl-1-naphthylamide, y-L-glutamyl-2-naphthylamide (both substances in concentration of 0.24 mg/1 ml of acetate buffer pH 6.5) were used as the substrates. Results were compared with those obtained with Fast Blue B and Fast Garnet GBC.

In comparison with Fast Blue B and Fast Garnet GBC HP is a faster coupler, furnishes azodyes which are stable, amorphous (even without lipid extractions from sections), more substantive and in the case of 1-naphthylamine almost insoluble in ordinary lipid solvents used for the dehydration and clearing of sections before mounting. The molecular extinction coefficient of azodyes furnished by HP is 1.5 × higher for 1-naphthylamine than for 2-naphthylamine. It is higher than that of Fast Garnet GBC, however, lower than that of Fast Blue B. The inhibitory influence of individual diazonium salts on enzyme activity (activities) splitting leucyl-2-naphthylamide amounts to 36% (Fast Garnet GBC), 37% (Fast Blue B), 52% (HP, 0.03 ml/1 ml) and 63% (HP, 0.09 ml/1 ml) at pH 6.5. For γ-glutamyl-transpeptidase the corresponding values are 50%, 59%, 62% and 67%. The higher inhibitory influence of HP is compensated by the possibility of its using in the technic of semipermeable membranes.

HP improves greatly the localization of peptidases in cold microtome sections from which lipids were not extracted. The best results are furnished by 1-naphthylamine derivatives. In the case of 4-methoxy-2-naphthylamine derivatives the localization is very sharp, however, the azodye is less distinct than that of 2-naphthylamine.

The localization as obtained with HP in combination with substrates derived of simple naphthylamines is similar or even better than with 4-methoxy-2-naphthylamine derivatives applied with Fast Blue B. Typical examples are shown.

Introduction

In the histochemical demonstration of peptidases azocoupling reactions play the most important role. In these reactions substrates derived of 2-naphthylamine (Gomori, 1954; Burstone and Folk, 1956; Nachlas et al., 1957; Glenner and Folk, 1961; Burstone, 1962; Glenner, 1962; Hopsu-Havu and Glenner, 1966; Hopsu; Havu and Eckfors, 1969), 1-naphthylamine (Albert et al., 1961, 1964, 1966; Hopsu-Havu et al., 1966) or 4-methoxy-2-naphthylamine (Nachlas et al., 1960; Monis et al., 1965; Rutenberg et al., 1969) are used. As coupling agents Fast

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Garnet GBC (Gomori, 1954; Burstone and Folk, 1956; Albert et al., 1961, 1964, 1966; Glenner and Folk, 1961; Burstone, 1962; Glenner, 1962; Hopsu-Havu and Glenner, 1966; Hopsu-Havu and Eckfors, 1969), Fast Blue B (Nachlas et al., 1957, 1960; Monis et al., 1965) or Fast Blue BBN (Rutenburg et al., 1969) have been recommended. Burstone (1962) considered Fast Garnet GBC the diazonium salt of choice. However, the azodye originating from it and 2-naphthylamine is soluble in lipids and crystallizes in sections from which lipids were not removed. Good results are therefore obtained only in paraffine sections of frozen dried, frozen substituted or of formol fixed tissue samples or in cold microtome sections which are frozen substituted or extracted with acetone at least (Lojda, 1970). Fast Blue B was preferred by Nachlas et al. (1957, 1960) and Monis et al. (1965) despite its higher inhibitory influence. This diazonium salt enables to use the chelation of the azodye, e.g. with cupric ions which makes the azodye more substantive. The azodye does not recrystallize. However, diffusion artifacts are inevitable when this diazonium salt is used in connection with substrates derived of unsubstituted naphthylamines. Therefore substrates derived of 4-methoxy-2-naphthylamine were recommended for a correct localization (Nachlas et al., 1960; Rutenburg et al., 1969).

Hexazonium-p-rosanilin (HP, introduced into histochemistry by Davis (1959) proved the diazonium salt of choice in the majority of azocoupling reactions (Lojda, 1970, 1972). Its use in connection with naphthylamine derivatives was mentioned by Albert et al. (1961, 1964) and by Monis et al. (1965). However, no advantage was reported (Albert et al., 1961, 1964) or results considered even less satisfactory than those obtained with Fast Blue B (Monis et al., 1965).

In our experiments with the histochemical demonstration of peptidases a great improvement in the localization was obtained when substrates derived of unsubstituted naphthylamines (particularly of 1-naphthylamine) were used with HP as the coupler. This combination proved very useful in connection with the demonstration of enzyme activities in sections adherent to semipermeable membranes. In this way any loss of enzyme activities due to inhibitory influence of the fixative used or to a leakage of soluble enzyme components into the incubation medium can be avoided. A critical evaluation of HP as the coupling agent in peptidase histochemistry is the subject of the present communication.

**Materials and Methods**

Pieces of rat kidney, jejunum, lung, epididymis, pancreas, and liver, of guinea pig jejunum as well as human enterobiopsies were quenched in petrolether chilled with acetone-dry ice mixture. Sections were prepared with a cold microtome and transferred to slides or semipermeable membranes as described previously (Lojda and Havráneková, 1975).

In histochemical experiments following substrates were used: Alanyl-1-naphthylamide (A1NA, kindly synthetized by Ing. E. Kasafirek, Research Institute of Pharmacy and Biochemistry, Praha, ČSSR), D,L-alanyl-2-naphthylamide (A2NA, Sigma Chem. Co., St. Louis, Mo., USA), L-leucyl-2-naphthylamide (L2NA, Koch-Light Labs., Colnbrook, Buckinghamshire, England), L-leucyl-4-methoxy-2-naphthylamide (LM2NA, Koch-Light), γ-L-glutamyl-1-naphthylamide (G1NA; kind gift of Doc. Dr. A. Szewczuk, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland), and γ-L-glutamyl-2-naphthylamide (G2NA, Lachema, Brno, ČSSR). These substrates were used either in standard incubation media (Lojda, 1970) with Fast Blue B (Lachema) or Fast Garnet GBC (Lachema) or in media with HP.