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Abstract

Immunodetection techniques are dependent on enzyme–protein conjugates for the visualisation of antigen–antibody complexes. One of the most widely used is the avidin–biotin–peroxidase complex (ABC) method. The present study demonstrates that direct treatment of goldfish and salamander retinal sections with ABC, followed by an incubation with the chromogenic substrate 3,3-diaminobenzidine tetrahydrochloride (DAB) and H_2O_2, manifested a punctate staining pattern across the neural retinae, presumably through binding of avidin to endogenous biotin. Incubation with a primary antiserum against biotin followed by immunoprocessing with the peroxidase–anti-peroxidase (PAP) method showed a pattern similar to the punctuate framework as detected with solo ABC-treated sections. Moreover, the ABC–DAB/H_2O_2 mediated pattern corresponded to the spatial orientation of Müller cells as identified by GFAP immunostaining. These findings indicate the presence of endogenous biotin in Müller cells and calls for caution in the application of the ABC method in immunotechniques in retinal research. © 1997 Elsevier Science B.V.

Keywords: ABC method; Biotin; Goldfish retina; Immunocytochemistry; Müller cells; Peroxidase–anti-peroxidase method; Salamander retina

1. Introduction

Biotin is a vitamin present in minute amounts in living cells. It is widely distributed in mammalian tissues and is present in relatively high concentrations in liver, adipose tissue, mammary gland and kidney (Dakshinamurti and Mistry, 1963; Moss and Lane, 1971; Wood and Borden, 1977); it plays an indispensable role in cellular carboxylation systems (Dakshinamurti and Chauhan, 1990). The extremely high affinity of avidin, a 68 kDa glycoprotein, for biotin has been effectively exploited for a variety of purposes, including quantitative enzyme immunoassays (Hsu et al., 1981a) and immunocytochemical localisation techniques (Wilchek and Bayer, 1982).

In immunoenzymatic techniques (Guedson et al., 1979; Hsu et al., 1981a,b; Fung et al., 1992), covalent coupling of biotin to immunoglobulins or peroxidase molecules renders them capable of binding avidin-labelled molecules. However, in tissues that are rich in endogenous biotin, an interpretation of the results of avidin–biotin-based immunodetection is difficult due to binding of free avidin, or avidin conjugates, to endoge-

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nous biotin (Hsu et al., 1981a; Wood and Warnke, 1981). While performing immunocytochemical studies on goldfish and salamander retina (Bhattacharjee et al., 1996) using the avidin–biotin–peroxidase complex (ABC) method, we observed a discrete staining pattern in control sections of the retina after omission of either the primary or secondary antibody, or both. A similar staining pattern was found in retinal sections treated directly with ABC followed by an incubation with a chromogenic substrate solution containing 3,3-diaminobenzidine tetrahydrochloride (DAB) and H_2O_2. These results suggest that avidin-like or biotin-like molecules are expressed in the retina, putatively in the retinal Müller cells (MC), which may contribute to an erroneous interpretation of retinal immunocytochemical results The present study was aimed at characterising the ABC-mediated staining reaction in goldfish and salamander retina.

2. Materials and methods

2.1. Animals

Juvenile (5–5.5 cm) and adult (6–16 cm) goldfishes (*Carassius auratus*) and salamanders (*Amblystoma mexicanum*, 15–22 cm) were anaesthetised with MS222 (Sigma, St. Louis, MO, USA) and decapitated, prior to enucleation of the eyes. Animal handling and anaesthetic procedures were reviewed and approved by the Committee for Animal Care and Use of the Faculty of Medicine of the University of Amsterdam.

2.2. Preparation of tissue

Following enucleation, the eyes were hemi-sected by an encircling cut around the limbus, the cornea and lens removed, and the vitreous partially drained with absorbent tissue. Eyecups were immediately fixed by immersion fixation in 4% paraformaldehyde (PFA), or 4% PFA (w/v)–0.05% glutaraldehyde (v/v), or 4% PFA (w/v)–15% saturated picric acid (v/v), made in 0.1 M sodium phosphate buffer (PB) at pH 7.2 for 4–6 h at 4°C. Eyecups were cryoprotected in 15% sucrose for 3 h, and in 30% sucrose overnight at 4°C. The following day, the tissue was embedded in Tissue Tek (Miles, USA), quickly frozen on dry ice, and either sectioned immediately on a cryostat or stored at −70°C. Transversely cut sections, 12–14 μm thick, were collected on chrome–alum–gelatin coated slides, dried for 2 h at room temperature, and stored at −20°C in Parafilm-wrapped small plastic slide containers.

2.3. ABC–DAB procedure

The sections were thawed for 30 min at room temperature in the plastic boxes and then postfixed in 4% PFA in PB for 10 min for better adhesion of the retinal sections on the glass slides. They were washed (4 × 10 min) in 0.1 M phosphate-buffered saline (PBS), and then treated in ABC (Vectastain Elite ABC kit, Vector, USA) solution for 1 h. The two reagents of the kit were mixed in a dilution of 1:50 in PBS and allowed to stand for about 30 min before use. This protocol is according to the instructions of the supplier. After ABC treatment and washing in PBS (3 × 10 min), the slides were treated either with 0.05% DAB (Sigma) and 0.01–0.03% H_2O_2 in PBS, or with a metal enhanced DAB Kit (Pierce, USA) for 10–15 min. The reaction was terminated by rinsing in PBS. After several washes in distilled water, the slides were dehydrated in ethanol, cleared in xylene and coverslipped in Entellan or Malinol. All washing procedures, the ABC incubation and DAB reaction steps were performed at room temperature under continuous agitation.

2.4. Immunohistochemistry

2.4.1. Primary antibodies

Mouse monoclonal antibodies, against biotin (anti-biotin; 1:3000, 1:4000 dilution), and against avidin (anti-avidin; 1:2000, 1:3000, 1:4000 dilution) in PBS containing 2% bovine serum albumin (PBS/BSA) were purchased from Sigma. An affinity purified peroxidase-labelled anti-biotin raised in goat was purchased from Vector and used at a 1:2000 or 1:3000 dilution in PBS/BSA. Polyclonal anti-GFAP (Fluka, Switzerland) was used in 1:200 or 1:300 dilution.

2.4.2. Immunolabelling protocol

The indirect peroxidase–anti-peroxidase (PAP) method (Sternberger et al., 1970; Sternberger, 1978) was used for the determination of avidin-, biotin- and GFAP-immunoreactivity. After thawing, postfixation and washing as described under the ABC–DAB procedure, the sections were further rinsed (1 × 30 min) in PBS/BSA and preincubated for 30 min in 5% normal rabbit serum (NRS, Nordic, Netherlands) for monoclonal antibodies, or 5% normal goat serum (NGS, Nordic) for the polyclonal antibodies. The sections were then incubated in primary antibodies, diluted in PBS/BSA, for 40–48 h in moist teflon trays at 4°C under agitation. After washing in PBS (4 × 10 min) and PBS/BSA (1 × 30 min), the sections were incubated for 30 min with either rabbit anti-mouse IgG (Nordic), or goat anti-rabbit IgG (Nordic), diluted 1:100 in PBS/BSA, containing respectively 1% NRS or 1% NGS. The wash sequences in PBS, PBS/BSA were repeated and incubation with either mouse peroxidase–anti-peroxi-

dase (mousePAP, Nordic) or rabbit peroxidase–anti-peroxidase (rabbitPAP, Nordic) diluted 1:200 in PBS/BSA for 1 h was carried out. After washing in PBS (4 × 10 min), the sections were treated with DAB/H_2O_2, washed, dehydrated and mounted. The biotin immunoreactivity in the retinae was also studied by a direct method, in which sections incubated with peroxidase-labelled anti-biotin (Vector), after washing, were directly subjected to DAB/H_2O_2 for visualisation of the antigenic sites. All secondary and tertiary level incubations and washing steps were carried out at room temperature (20°C) under continuous agitation.

2.4.3. Specificity controls

To demonstrate the specificity of the immunohistochemical staining, controls were run either by (i) omission of primary antibodies, (ii) omission of secondary antibodies, (iii) omission of both primary and secondary antibodies, or (iv) a series of blockage experiments to test whether any staining occurred following specific blockage of endogenous avidin or biotin binding sites. For the latter, parallel sections were incubated with anti-biotin (1:1000–1:4000), anti-avidin (1:2000–1:4000) for 48 h at 4°C, followed by the ABC–DAB procedure. Furthermore, sections were treated with avidin (1:2000; Sigma, USA) for 1 h at 20°C followed by the ABC–DAB procedure. (v) Finally, a direct method for the detection of endogenous biotin was employed by incubating retinal sections with peroxidase-labelled avidin (A-HRP; 1:1000–1:4000; Bio-Rad, USA) for 1 h at 20°C and after washing treated with DAB.

2.5. Western blotting

The retinae of two goldfishes were isolated from the pigment epithelium and homogenised in 0.32 M sucrose, 1.0 mM EDTA, 0.25 mM dithiothreitol, 0.2 mM phenylmethylsulfonylfluoride (PMSF), pH 8.0. After centrifugation (15 000 × g, 30 min at 4°C), the membranes were solubilised in 20 mM Tris–HCl, 1% Triton X-100, 0.2 mM PMSF (pH 8.0) for 1 h at 4°C. Unsolubilised proteins were removed by centrifugation and the protein content of the samples was measured (Bio-Rad protein assay). Protein samples were diluted in a buffer (pH 8) containing Tris (62 mM), EDTA (1 mM), SDS (1%), dithiothreitol (1%) and boiled for 5 min before loading on the gel. Biotinylated marker proteins were used as molecular mass standards (Bio-Rad). Western blot analysis of the homogenates was performed after fractionating 0.5 μg total protein by 10–15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (PhastSystem-Pharmacia) and a subsequent transfer to nitrocellulose membrane (Hybond ECL-Amersham) by electroblotting.

The presence of biotinylated proteins on the membrane was visualised using an avidin–HRP conjugate (Bio-Rad). The blot was incubated with a dilution of 1:750 000 of the conjugate for 1 h at room temperature, followed by chemiluminescence detection using an ECL-kit (Amersham, England) according to the manufacturers instructions. A separate lane of the blot was incubated with anti-biotin (1:6000) at 4°C for 16 h, followed by detection as described above.

3. Results

Using the ABC system in our immunocytochemical research on the retina of goldfish and salamander, we consistently observed in control sections without primary or secondary antibody treatment, a labelling pattern that resembled the columnar organisation of the Müller cells (Fig. 1a and Fig. 2a). Remarkably, this pattern was still found after omission of the primary and secondary antibody incubation steps from the protocol, whereas no staining was revealed after treating the sections with DAB/H_2O_2 alone, ruling out endogenous peroxidase as the source for the staining. The use of a StreptABC kit yielded similar results as the Vectastain Elite ABC kit. Replacing the ABC incubation for a mousePAP or rabbitPAP step resulted in completely blank sections, again pointing out the dependence of the staining on the ABC incubation. The use of different fixatives, PFA, PFA/glutaraldehyde, or PFA/picric acid, did not lead to any significant differences in the ABC- or biotin-immunostaining. Similarly, use of 0.1 M Tris buffer, with or without 0.1% Triton X-100 instead of PBS, did not affect the staining reaction. In the following sections, we describe (i) the pattern of staining resulting from the ABC–DAB incubation, (ii) the presence of biotin immunoreactivity in goldfish and salamander retina, (iii) a series of experiments to verify the dependence of the ABC-mediated staining on the presence of endogenous biotin, (iv) the distribution of the Müller cell marker GFAP in the retina, and (v) the results of Western blotting experiments.

3.1. Pattern of staining manifested by ABC–DAB treatment

Fig. 1a illustrates the pattern of staining obtained in the adult goldfish retina following treatment with ABC and DAB, without any primary or secondary antibodies. The staining reaction was restricted to the photoreceptor ellipsoid of the inner segment and the vertically orientated punctuate processes extending from the vicinity of the outer limiting membrane (OLM) to the inner limiting membrane (ILM). The stained processes appeared thick and evenly spaced at the levels of the inner nuclear layer (INL) and ILM, but were less
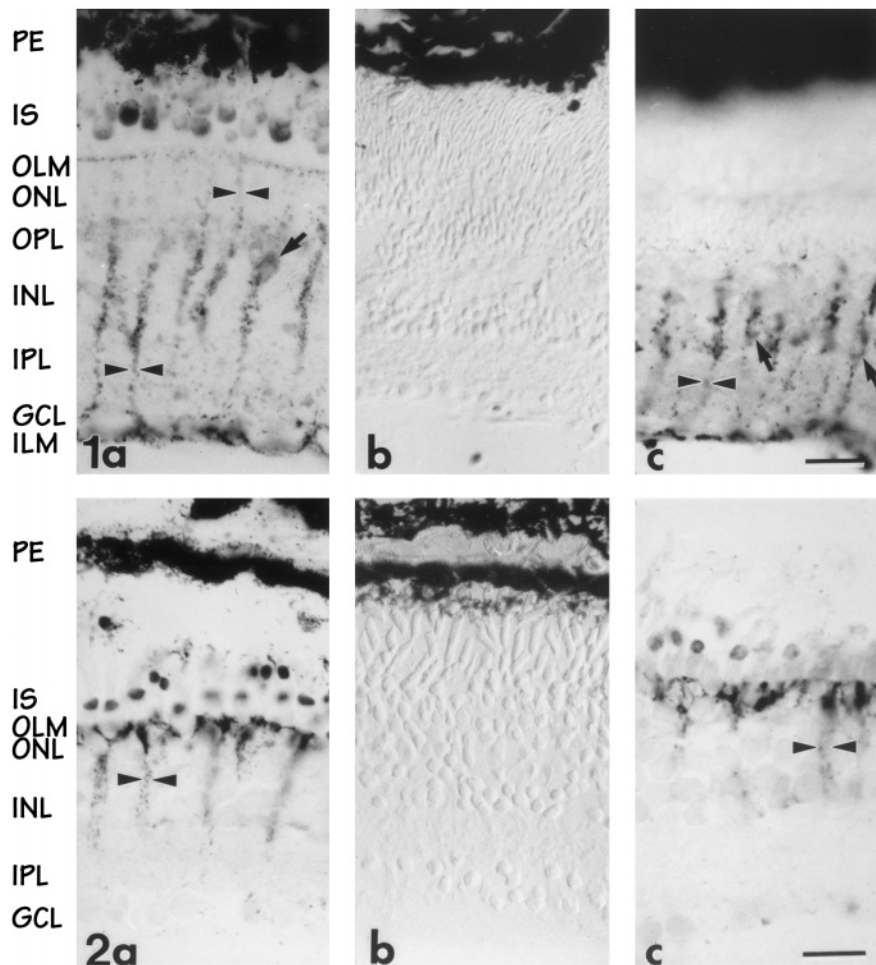
Fig. 1. Comparison of staining after ABC–DAB procedure (a), with that of anti-avidin immunoreactivity, visualised by the PAP method (b) and anti-biotin PAP immunoreactivity (c), in goldfish retina. Note the similarities in staining reactions between lane a (ABC–DAB) and lane c (anti-biotin), and the absence of staining reaction in lane b (anti-avidin). GCL, ganglion cell layer; ILM, inner limiting membrane; INL, inner nuclear layer; IPL, inner plexiform layer; IS, inner segment ellipsoid; OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; PE, pigment epithelium. Arrows indicate diffuse perinuclear staining in (a) and (c). Arrowheads indicate position of radial fibers of Müller cells. Bar represents 50 μm.

Fig. 2. Comparison of staining after ABC–DAB procedure (a), with that of anti-avidin immunoreactivity, visualised by the PAP method (b) and anti-biotin PAP immunoreactivity (c), in salamander retina. Note the similarities in staining reactions between lane a (ABC–DAB) and lane c (anti-biotin), and the absence of staining reaction in lane b (anti-avidin). Abbreviations as in Fig. 1.

discrete at the level of the inner plexiform layer (IPL). Although hardly any staining could be localised at the level of the outer nuclear layer (ONL), discrete staining resulted at the level of the OLM, following treatment of ABC-reacted slides with metal enhanced DAB. Visualisation of the ABC-mediated staining by metal enhanced DAB also resulted in sharper staining in the photoreceptor ellipsoids, and the outer plexiform layer (OPL) was also stained in juvenile goldfish. However, the punctuate vertical processes in the retina of juvenile fishes were numerically less than their adult counterparts.

ABC treated salamander retina (Fig. 2a) yielded a pattern of staining similar to that of the goldfish (compare Fig. 2a and Fig. 1a). Dense reaction products were localised at the level of the OLM. The reaction product,

distributed perpendicular to the retinal layers in a thick columnar form, was, however, finer and more homogeneous. The photoreceptor ellipsoids of the inner segment showed a discrete staining reaction. The photoreceptor outer segments, nuclei in the ONL, somas in the INL and ganglion cell layer (GCL), and the ILM, remained unstained.

3.2. Biotin immunoreactivity in goldfish and salamander retina

To determine the nature of this ABC-dependent staining, the distribution of avidin and biotin immunoreactivity was studied in both goldfish and salamander retina using specific antibodies against avidin or biotin, followed by PAP-based detection of im-

munoreactivity. Whereas no labelling could be found for avidin (Fig. 1b and Fig. 2b), strong signals for biotin were recorded in the retinae as shown in Fig. 1c and Fig. 2c. The biotin immunoreactivity in goldfish and salamander retinae was similar to that after ABC-mediated staining reaction (compare Fig. 1a and c). Goldfish or salamander retina incubated with peroxidase-labelled anti-biotin followed by DAB yielded an identical pattern of staining (results not shown).

3.3. Blockage experiments

The absence of avidin immunoreactivity and the discrete localisation of biotin labelling in the retinae strongly indicated that endogenous biotin may be the key element in the ABC-dependent staining. To confirm this hypothesis, a series of blockage experiments was carried out. (i) The putative binding sites of endogenous biotin and avidin were blocked in parallel sections, respectively by anti-biotin and anti-avidin antibodies, followed by ABC and DAB treatment. Whereas incubation with anti-biotin produced a reduction of the staining intensity, the discrete ABC-mediated staining pattern was not decreased in sections incubated with anti-avidin. (ii) Blockage of endogenous biotin by avidin, followed by ABC and DAB, yielded no staining. (iii) Incubation of sections with peroxidase-labelled avidin (A-HRP; Bio-Rad, USA) followed by DAB did not result in the typical ABC-staining pattern, presumably related to the lower sensitivity of this method. Results of the various sets of experiments carried out to characterise the nature of ABC staining in goldfish and salamander retina are summarised in Table 1.
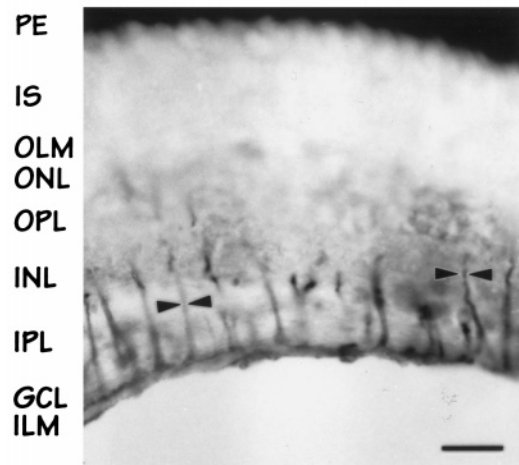

Fig. 3. GFAP immunoreactivity in goldfish retina. Compare the organisation of the radially disposed processes of Müller cells with that of Fig. 1a and c. Arrowheads indicate position of radial fibers of Müller cells. Abbreviations as in Fig. 1. Bar represents 50 μm.

3.4. GFAP immunoreactivity

Since the ABC–DAB staining pattern resembled the organisation of retinal Müller cells (MC), we used a specific marker for the cytoskeletal elements of astrocytes, to compare this pattern with the ABC–DAB pattern. In goldfish, the immunolabelling obtained with anti-GFAP was restricted to the radial processes of MC, extending between the OLM and ILM. The perikarya of MC did not express any detectable signal. Fig. 3 shows the disposition, including the regularity in the columnar distribution of the labelled MCs. Whereas long and articulate internal filamentous processes extending between the INL and ILM were clearly evident, the external processes at the level of the INL and the OLM were comparatively less well defined in our preparations. The overall staining pattern corroborates the earlier observations (Bignami, 1984; Jones and Schechter, 1987) involving GFAP labelling in goldfish retina. No discrete GFAP immunostaining could be obtained in salamander retina.

3.5. Western blot analysis

Fig. 4 shows the result of biotin immunostaining with avidin–HRP on blotted retinal proteins of goldfish, revealing a distinct band at about 120 kDa. However, when the proteins on the membrane were preincubated with the monoclonal antibody against biotin, to ascertain whether detection of endogenous biotin by the avidin–HRP conjugate could be blocked, complete suppression of this band resulted. This finding corroborates the results of similar experiments carried out on retinal sections, by employing the same batch of monoclonal antibody.

Table 1
Detection ($+$), partial suppression ($\pm$), and complete suppression/absence ($-$) of endogenous staining pattern in goldfish and salamander retina

Treatment	Goldfish	Salamander
ABC–DAB	+	+
DAB	−	−
Avidin–ABC–DAB	−	−
Avidin∼HRP–DAB	−	−
Anti-biotin–rabbit–anti-mouse –mousePAP–DAB	+	+
Anti-biotin∼PO–DAB	+	+
Anti-avidin–rabbit–anti-mouse –mousePAP–DAB	−	−
Anti-avidin–ABC–DAB	+	+
Anti-biotin–ABC–DAB	±	±

DAB, DAB solution containing 0.01–0.03% H_2O_2; ABC, avidin–biotin–peroxidase complex; ∼HRP, horseradish peroxidase labelled; ∼PO, peroxidase labelled; PAP, peroxidase–anti-peroxidase.

4. Discussion

4.1. Rationale of ABC staining reaction

In diagnostic pathology and immunocytochemical research, immunoenzymatic techniques are extremely valuable. Many immunoenzymatic staining methods are currently available for localising tissue antigens, and all the methods necessitate treatment of immunoprocessed tissues with a suitable chromogenic substrate for visualisation of antigenic sites. The avidin–biotin–peroxidase complex (Hsu et al., 1981a,b) has proven to be one of the most versatile detection systems in immunocytochemistry (Wilchek and Bayer, 1982). This system utilises the exceedingly high affinity of avidin or streptavidin for biotin, and requires incubation with a biotinylated primary or secondary antibody prior to treatment with preformed ABC, or enzyme-labelled avidin. During the ABC reaction stage, open sites on avidin from ABC reagent or enzyme-labelled avidin bind to the biotinylated antibody. Since in most tissues biotin concentrations are low, the ABC methods are commonly used in immunocytochemical studies. However, in the present study we have shown that the ABC complex stains specifically MC in goldfish and salamander retina, probably due to a high biotin store in these cells. The discreteness of the staining resulted from only an incubation with ABC followed by DAB reaction, and the absence of staining with singular treatment of DAB solution, lead to the conclusion that the staining emanates from the ABC stage alone. This finding
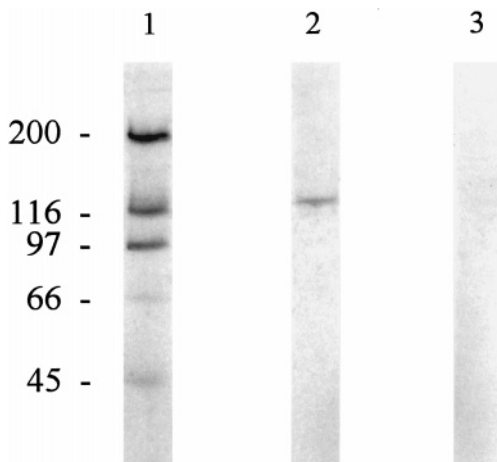

Fig. 4. Tissue homogenate of goldfish retina was subjected to electrophoresis and blotted to nitrocellulose membrane. After transfer to nitrocellulose membrane, the presence of biotinylated protein was visualised by incubation with an avidine–HRP conjugate followed by chemiluminescence detection of the HRP activity. Lane 1: biotinylated molecular mass standards, sizes (in kDa) are shown on the left. Lane 2: protein homogenate (0.5 μg) of goldfish retina. A distinct band corresponding to a size of about 120 kDa is revealed. Lane 3: preincubation with anti-biotin followed by avidin–HRP incubation. The detection of the band visualised in lane 2 is completely blocked.

makes the use of ABC methods for these preparations problematic.

Retinae of various vertebrate species including goldfish (Peng et al., 1995) and salamander (Liepe et al., 1994; Yang and Yazulla, 1994) have been examined during the last couple of years by ABC techniques. However, interference of a MC-like staining pattern in the neural retina by endogenous biotin, as presented in this paper, has not yet been reported, although a Texas Red-Avidin staining in the MC in carp retina has been described (Teranishi and Negishi, 1994). Since the findings reported here of an ABC-mediated staining pattern were initiated by a conventional series of control experiments, it is unclear why this effect has not been encountered or recognised as a problem heretofore. Possibly, some recent improvements in the detection sensitivity of the available ABC kits may be an explanation. The observation that incubation with avidin–HRP does not lead to a significant staining pattern suggests that the greater sensitivity of the ABC complex, induced by the presence of multiple peroxidase molecules per endogenous biotin site, plays a key role.

4.2. Pseudo-immunocytochemical reaction with ABC versus biotin immunoreactivity in the retina

Fig. 1a and Fig. 2a demonstrate the punctuate and fibrillar distribution of the chromogenic reaction products in the goldfish and salamander retina, respectively, following ABC and DAB treatments. The biotin immunoreactivities obtained by use of both unlabelled anti-biotin and peroxidase-labelled anti-biotin, without any intervening ABC stage, were almost identical to the non-immunohistochemical reactions manifested by ABC (compare Fig. 1a and Fig. 2a with Fig. 1c and Fig. 2c). The regularity with which these vertically disposed stained entities occur along the depth of the retinae studied, was virtually identical to the classical profile of the MC as described and illustrated in various vertebrate species (Polyak, 1941; Cajal, 1972). Masking of biotin sites through preincubation with anti-biotin reduced the number of free biotin sites available where avidin or avidin conjugates could otherwise bind, resulting in a suppression of the staining intensity in sections treated with ABC and DAB (see Table 1). Moreover, the immunoblot experiments revealed a clear band after incubation with avidin–HRP while this pattern could be blocked by a pre-treatment with anti-biotin. Taking all evidence into consideration, we conclude that the ABC staining recorded in goldfish and salamander retina is derived from binding of avidin from ABC to endogenous biotin. Similar phenomena were recorded in autopsied human liver and kidney, and were referred to as endogenous avidin binding activity (Wood and Warnke, 1981). The comparable pattern of distribution of reaction products between

anti-GFAP, and anti-biotin and ABC–DAB preparations, strongly suggest MC to be the principal source of biotin in the goldfish retina (Bignami, 1984; Jones and Schechter, 1987). Although we were unable to obtain discrete anti-GFAP activity in the retinal MC in salamander, the disposition of MC, stained by anti-vimentin and NADPH in tiger salamander retina (Liepe et al., 1994) corroborated the disposition of the ABC-stained and biotin-immunoreactive cell profile demonstrated in salamander. These results support our conclusion that avidin binding from ABC in the retinae studied was due to the presence of endogenous biotin localised primarily in MCs.

The observed predominant cytoplasmic localisation of biotin is consistent with the intracellular distribution of enzymes for which biotin is a known prosthetic group (Levy et al., 1978; Wood and Warnke, 1981; Duhamel and Whitehead, 1990). Biotin-containing cytoplasmic binding sites for avidin have been identified in kidney, liver, pancreas (Wood and Warnke, 1981; Rowley and Eisenberth, 1982) and brain (Naritoku and Taylor, 1982; Levin and Macklin, 1988). Moreover, in the context of past biochemical studies of a predominantly mitochondrial localisation of biotin–C^{14}OOH in rat and chick liver (Dakshinamurti and Mistry, 1963), a mitochondrial localisation of biotin across the radial expanse of MC may be possible. A localisation of biotin in the photoreceptor ellipsoids is also consistent with the mitochondrial load, the region is known to be bestowed with. However, definitive proof of a mitochondrial localisation of biotin in MCs can only come from EM studies.

The result of the present study demonstrates the usefulness and inherent values of running appropriate controls, so as to avoid possible misinterpretations of results, especially while employing ABC techniques. An easy way to combat such false positivity due to endogenous biotin activity could be to incubate tissues with free avidin before the ABC stage, as also proposed by Wood and Warnke (1981).

Acknowledgements

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