Detection of γ-Crystallins in the Developing Amphibian Lens by Peroxidase-Labelled Antibodies

Fluorescent antibodies are widely used to trace the appearance of specific proteins during cell differentiation. In this report an alternative method of labelling antibodies, i.e., conjugation to horseradish peroxidase was tested for such a purpose. In contrast to the fluorescent label, which can be visualized only by a fluorescence microscope, peroxidase reacts with H_2O_2 and diaminobenzidine[1] to produce dense material which is detected in the transmitted light and in electron microscopes. We have compared the ability of fluorescein isothiocyanate-labelled and peroxidase-labelled anti-γ crystallins to detect γ-crystallins in developing and regenerating amphibian lenses.

Material and methods. The normal lens rudiments of *Rana pipiens* at lens stages VIII to X[2] and lens regenerates of adult *Notophthalmus* (*Triturus*) *viridescens* at stages VIII to X[3] served as assay systems. Specific antibodies against γ-crystallins of *Rana pipiens* were prepared as described by McDevitt et al.[2]. Goat antirabbit γ-globulins (GAR) were purchased from commercial sources (Antibodies, Inc. and Immunology, Inc.). Most of the following procedures were carried out in a cold room at 3–4 °C. Conjugation of antibodies to horseradish peroxidase was done by the procedure of Nakane and Pierce[4] with some modifications. Commercial GAR was dialyzed against 4 changes of 200 volume of 0.5 *M* carbonate buffer ($Na_2CO_3/NaHCO_3$, pH 10.0) for 16–24 h. A solution of 300 mg of GAR and 300 mg of peroxidase (Type II, Sigma Chemical Co) in 12 ml of carbonate buffer was made. Conjugation was achieved by slowly adding to this solution 7.5 mg of the coupling agent, *p,p'*-difluoro-m,m'-dinitro-diphenyl sulfone (General Biochemicals), dissolved in 1.5 ml of cold acetone with constant stirring. A slight precipitate formed. The reaction mixture was gently stirred for 4.5 h, dialyzed against 100 volumes of phosphate buffered saline (PBS) for 12 h, and centrifuged at 12,100 *g* (Sorvall, Rotor SS-34) for 15–20 min. The supernatant now contained 'denatured' proteins, conjugated globulins, unconjugated globulins, and free peroxidase. The latter was removed by precipitating the globulins with 50% sat. $(NH_4)_2SO_4$. The precipitated globulins were redissolved in 5–6 ml of PBS and passed through a Bio-Gel P-300 column (100–200 mesh, Bio-Rad Corporation), equilibrated with PBS. The fractions were collected as 2.8 ml aliquots and read on a spectrophotometer at 280 nm and at 403 nm, the absorption maximum for peroxidase (Figure 1).

Tissues were fixed in 4% formaldehyde, dehydrated and embedded in Tissuemat (Fisher Scientific) according to Nöthiger et al.[5]. Fluorescent antibodies (GAR conjugated to fluorescein isothiocyanate, FITC) were applied as described by Nöthiger et al.[5], using the 'indirect method' of Weller and Coons[6] with specific antibodies against *Rana pipiens* γ-crystallins as the intermediate

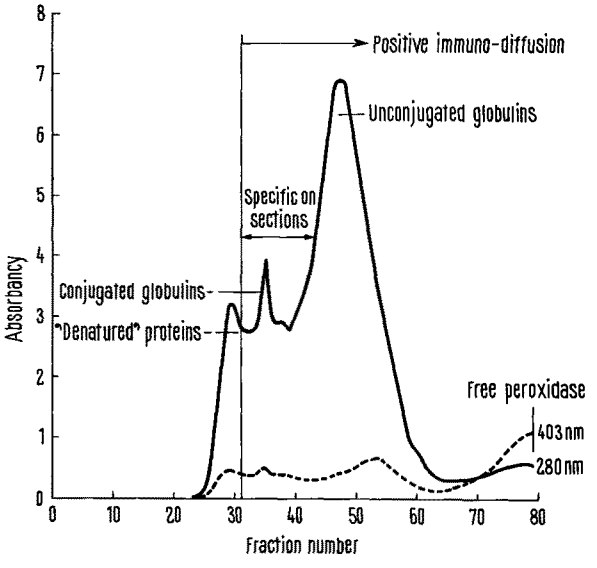

Fig. 1. Separation of conjugated antibodies: chromatographic pattern of the reaction mixture after conjugation of GAR to peroxidase. Bio-Gel P-300, column length 53 cm, diameter 3 cm, flow rate 2–5 ml per h, fractions collected as 2.8 ml aliquots. —, 280 nm; – – –, 403 nm. 'Denatured proteins': globulin-globulin complexes, overlabelled globulins, etc.

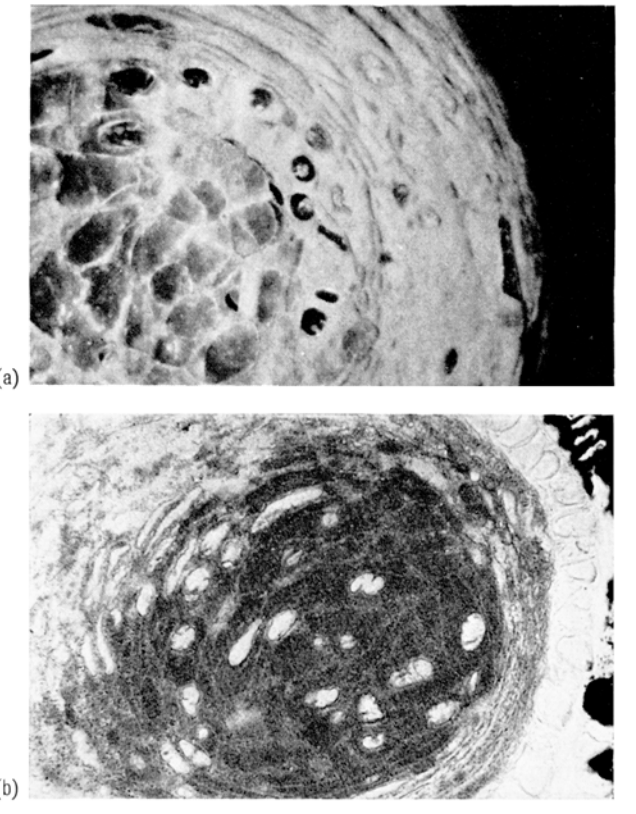

Fig. 2. Lens regenerate of *N. viridescens*, treated with *R. pipiens* anti-γ crystallin, and stained with FITC-GAR (a), or peroxidase-GAR (b). Labelling in both cases was restricted to the lens fiber area. In (a) the label is bright, the background dark; in (b) the label is dark, the background bright. The lens epithelium to the right is negative in both (a) and (b).

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layer. The peroxidase-labelled GAR was applied for 60–90 min to the sections pretreated with the above antibodies. After three 20 min washes with PBS, the sections were reacted with diaminobenzidine (DAB[7]) for 20–30 min. The reaction and the last wash with PBS occurred at room temperature. The sections were then rinsed and washed in 3 changes of distilled water for 3–5 min each and mounted in Elvanol[8]. Controls consisted of sections treated with non-immune rabbit γ-globulins as an intermediate layer.

Results and conclusions. The chromatographic pattern shown in Figure 1 has been reproduced 4 times. The procedure results in a satisfactory separation of conjugated globulins from the rest of the reaction mixture. This was demonstrated by the following tests: 1. Agar immunodiffusion revealed cross reaction with rabbit γ-globulins for fractions 31–52 of Figure 1; 2. When the same fractions were applied to sections of *Rana pipiens* embryonic lenses, pretreated with specific antibodies against γ-crystallins, specific staining in the lens fiber area was found only for fractions 31–43, being most pronounced for fractions 35–41. Therefore fractions 31–41 were pooled and absorbed 3 times with mouse tissue powder (20 mg, each time). After centrifugation at 12,100g for 1 h the resulting 25 ml of supernatant were dialyzed against 4 changes of 50 volumes of distilled water for 8 h and then lyophilized. The yellow powder obtained can be kept indefinitely under vacuum in the cold. For use, it is dissolved in PBS (6–8 mg/ml). This solution shows strong cross reaction with rabbit non-immune 7 S γ-globulins (0.5 mg/ml) in OUCHTERLONY tests[9].

Sections through lenses of *R. pipiens* or *N. viridescens* regenerates stained with peroxidase-labelled antibodies show that γ-crystallins are present in the fiber cells, but absent in the lens epithelial cells. This finding confirms previously published results[2,10]. Thus, peroxidase as a label for antibodies gives the same results as does an FITC label (Figures 2 and 3)[11].

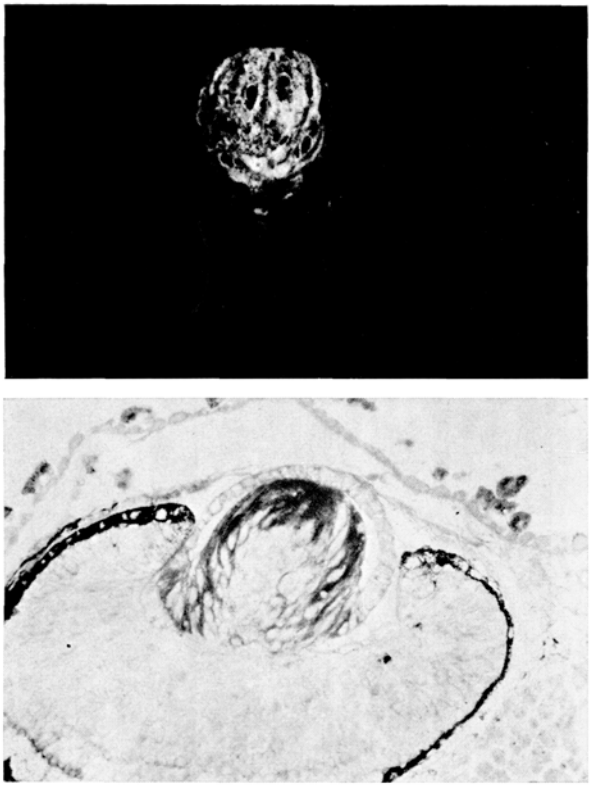

Fig. 3. Embryonic lens of *R. pipiens* (stage IX/X) treated with *R. pipiens* anti-γ crystallin, and stained with FITC-GAR (a), or peroxidase-GAR (b). Labelling in both cases was restricted to the lens fiber area. In (a) the label is bright, the background dark; in (b) the label is dark, the background bright. The black area in (b) is the pigmented layer of the optic cup.

Zusammenfassung. Antikörper, die entweder mit Fluoresceinisothiocyanat oder mit Peroxidase markiert waren, wurden auf ihre Spezifität im Erkennen von Linsen-Antigenen untersucht. Beide Markierer ergeben gleiche Resultate: die γ-Kristalline können in den Linsenfaserzellen lokalisiert werden, nicht aber in den Linsenepithelzellen.

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[7] 3,3'-diaminobenzidine (Sigma Chemical Company): 50 mg of DAB in 100 ml of 0.05 *M Tris*-HCl buffer, pH 7.6, 0.001% H_2O_2 were used as reagent for peroxidase.

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Mitotic Karyotype and Nucleoli of the Marbled Newt *Triturus marmoratus* (Latreille)[1]

Triturus marmoratus (Latreille, 1800) is an European newt species confined to the Iberian Peninsula and southwestern France. It can be grouped with *T. cristatus* as a superspecies or Artenkreis[2]: in fact they are 2 related species[3] that give rise to the natural hybrid *T. blasii* de l'Isle (1862) in the region in France in which their ranges overlap. Such area is rather small compared with the ranges of both species: *T. marmoratus* and *T. cristatus* can therefore be regarded as allopatric in the sense of MAYR[4], apart from the zone of overlap and hybridization.

The phenotypical and anatomical characters of *T. marmoratus*, *T. cristatus* and *T. blasii* have been widely dealt

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