A Method for the Demonstration of NADPH-Diaphorase Activity in Anuran Species Using Unfixed Retinal Wholemounts*

Robert GÁBRIEL**

Department of Anatomy and Histology, The Flinders University of South Australia School of Medicine, Adelaide, SA, Australia

Received January 12, 1991

Summary. The presence of NADPH-diaphorase enzyme has been previously revealed in fixed mammalian retinal tissue (SAGAR, 1986). Fixed retinas of *Bufo marinus* and *Xenopus laevis* failed to yield selective staining when reacted for NADPH-diaphorase. Satisfactory staining of retinal neurons was attained when the histochemical reaction was carried out in unfixed retinal wholemounts.

The applied method included the following steps: 1) Dissection of the fresh retina and the separation of the neural retina from all other coats of the eye ball, including the vitreal tissue; 2) pretreatment with 300 mM sucrose in phosphate buffer; 3) incubation with NADP, malic acid and nitroblue tetrazolium in phosphate buffer (pH 7.6); and 4) fixation of the tissue in 10% buffered formaldehyde overnight followed by whole mounting.

For control, fixed and unfixed rabbit and human retinas were also reacted for NADPH-diaphorase according to the above method. In these species specific staining was achieved only with fixed tissues. The possible implications of revealing NADPH-diaphorase enzyme activity in fixed mammalian and non-fixed anuran tissues are discussed.

Nicotinamide-adenine-dinucleotide-phosphate diaphorase (NADPHd) staining results in a Golgi-like image of the reacting nerve cells. The original histochemical procedure of SCHERER-SINGLER et al. (1983), has been applied to sections of the mammalian striatum first. SAGAR (1986) has modified this technique for retinal wholemount studies in the rabbit. He used short paraformaldehyde fixation then malic acid that served as hydrogen donor for reducing NADP to NADPH by an endogenous dehydrogenase enzyme. Finally nitroblue tetrazolium (NBT) was used as a chromogen to gain a suitable dark blue formazan endproduct. Most of the recent studies (see WÄSSLE et al., 1987; VANEY and YOUNG, 1988; MITROFANIS, 1989; SATO, 1990a) used this method to characterise NADPHd-containing nerve cells in the retinae of different mammalian species. Selective, but weak amacrine cell staining has been obtained recently with the SAGAR (1986) method in sectioned retina in *Rana esculenta* (SATO, 1990a). However, this method did not yield satisfactory results in fixed retinal wholemounts or transverse sections in two anuran species, *Bufo marinus* and *Xenopus laevis*. The aim of this study was to modify the original SAGAR (1986) method in order to stain NADPHd containing neurons in wholemounted preparations of the anuran retina. It is shown that a strong NADPHd staining of amacrine and bipolar cells can be achieved in unfixed anuran retinal tissues.

MATERIALS AND METHODS

Retinal tissues of adult *Bufo marinus* (9), *Xenopus laevis* (4), rabbit (3) and pieces of 3 human retinas (3–4 h postmortem) were used in this study. Animals were sacrificed by an overdose of anaesthetics (anura: MS 222; rabbit: nembutal), the eyes were removed and the neural retina was carefully dissected and separated from the other coats of the eye ball, including the vitreous in 0.1 M phosphate buffer (PB; pH 7.4).

---

*This work was supported by a National Health and Medical Research Council of Australia Grant to Dr. C. STRAZNICKY.

**R. G. was Visiting Research Scholar, on leave from Department of Zoology, Attila József University, Szeged, Hungary.
Neural retinae were either fixed using 3 different fixatives:
1) 4% paraformaldehyde in 0.1 M PB (pH 7.4),
2) 4% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M PB (pH 7.4),
3) 2% paraformaldehyde, 0.2% picric acid in 0.1 M PB (pH 7.2),
4) or used unfixed in each of the 4 species.

Four fixation schedules were applied in all experiments (1 h, 30 min, 15 min and 5 min).

After fixation the retinae were washed in 0.1 M PB or 0.01 M salinated PB for 4-8 h, then transferred to same solution containing 300 mM sucrose and 0.2% Triton X 100 for 5-10 min.

Composition of reaction mixtures: All the mixtures contained 5 mg NBT as a chromogen. The final volume of the reaction mixtures was 20 ml and that contained the following:

1) 10 mg NADP, 10 mg malic acid, 0.2% Triton X 100 in 0.1 M PB (pH 7.6).
2) 10 mg NADP, 10 mg malic acid, 0.2% Triton X 100, 0.2 mg manganese chloride in 0.1 M Tris buffer (pH 7.8).
3) 10 mg NADP, 10 mg malic acid, 0.2% Triton X 100 and 0.2 mg manganese chloride in tissue culture medium (pH 7.2; WOLF and QUIMBY, 1964).
4) 10 mg NADPH, 0.2% Triton X 100 in 0.1 M PB (pH 7.6). All chemicals used in the reaction mixtures were obtained from Sigma. The third solution was used for the incubation of the fresh tissues only. The duration of the incubations was in general for 3 h, but when the staining, viewed under microscope, was not considered optimal, the reaction time was extended up to 24 h.

Fig. 1. NADPHd reactivity in rabbit and human retina. a. The cell types (arrows and arrowheads) are clearly visible in rabbit retina after 4% paraformaldehyde fixation. Note the extremely dense dendritic branching. b. Type one cell can be found in the 4% paraformaldehyde fixed human retina with fine, long dendrites. Arrows perikarya. c. Human retina. 0.1% glutaraldehyde in the fixative reduces the background staining but decreases the dendrite labelling at the same time. Arrows: stained perikarya. d. Unfixed human retina. The reaction is not confined to any specific cell type. bv Blood vessels. Bars in a and d=50 μm; in b and c=60 μm.
After incubation the fixed tissues were rinsed in 0.1 M PB for 2 times for 5 min and the unfixed retinae were fixed in 10% buffered formaldehyde for at least 12 h, then rinsed 2 times for 5 min in 0.1 M PB. Radial cuts were made on the retinal wholemounts and they were flatmounted on 4% gelatin coated slides the ganglion cell layer lying uppermost and mounted in buffered glycerol (pH 8.6).

Controls: Omission of the NADP or NBT from the incubating mixtures abolished all specific staining. Excluding Triton X 100 from the incubating mixture slowed the development of the reaction.

RESULTS

After 1 h paraformaldehyde fixation, the original Sagar method (reaction mixture 2) gave similar results to those previously published in rabbit (Sagar, 1986; VaneY and Young, 1988) and in human (Sandell, 1985; Provis and Mitrofanis, 1990). In rabbit, two different amacrine cell populations were clearly seen (Fig. 1a), while in the human retina, a uniformly distributed amacrine cell population was demonstrated (Fig. 1b). In contrast, no NADPHd

![Fig. 2. NADPHd staining in the anuran retina. a. Bipolar cells (arrowheads) at the edge of an unfixed Bufo retina. b. Bipolar cell somata (arrowheads) in an unfixed wholemount of Xenopus retina. c. Stained amacrine cell (open arrow) amongst the labelled bipolar cells (arrows) in Bufo retina. d. Stained amacrine cell (arrow) from the unfixed Xenopus retina. e. Xenopus retina. Nonspecific staining in the photoreceptors after fixation and 20 h incubation. No other cell types have been stained. Bars in a, b, d = 20 μm; in c = 50 μm; in e = 100 μm.](image)
staining was observed in the fixed anuran retinal wholemounts treated in the same way, even after a day long incubation. With decreasing fixation time, the mammalian tissues retained their reactivity, however, the nonspecific background became higher. In the anuran retinae, photoreceptor cells stained slightly, after less then 30 min fixation, although no other cell types showed specific staining (Fig. 1c).

Glutaraldehyde (0.1%) added to the fixative, decreased but did not abolish the specific staining (Fig. 1c), although the non-specific background reactivity was greatly reduced. Picric acid in a concentration as low as 0.2%, completely destroyed the enzyme activity. Comparable good results can be achieved in the mammalian tissues following 4% paraformaldehyde fixation, provided the incubation takes place in 0.1 M PB. In this case manganese chloride is to be omitted from the incubating mixture (see incubating mixture 1), because the manganese ions form insoluble precipitate with the phosphate ions.

NADP and malic acid could be replaced with NADPH in the incubating medium (incubating mixture 4) which resulted in faster but poorer staining quality. The nonspecific background activity, however, became much higher, and NBT started to precipitate from the solution as did formazan after half an hour. Therefore, frequent changes of the incubating medium was required.

When unfixed tissues were used no specific staining was revealed in the rabbit and human retinae with any of the above described incubating media (Fig. 1d). In contrast, good quality staining was achieved both in the *Bufo* and *Xenopus* retinae with reaction mixture 1. The specific staining included bipolar (Fig. 2a, b) and amacrine cells (Fig. 2c, d). The morphological characterisation and the retinal distribution of these cells in the *Bufo* retina will be described in a separate paper (STRAZNICKY and GÁBRIEL, 1991).

It is worthwhile noting that if the 300 mM sucrose pretreatment was omitted, the quality and the specificity of the staining deteriorated. No staining was achieved with reaction mixture 2 and 3, and only weak reaction was obtained after incubation in mixture 4, with an accompanying very high background staining.

**DISCUSSION**

Although the NADH/NADPHd reaction is widely used for staining and characterisation of certain neurons both in the central and peripheral nervous system of various vertebrate species (SANDELL, 1985; VINCENT, 1986; GABELLA 1987; GABALLA and HALASY, 1987; MIZUKAWA et al., 1989; SATO, 1990b) the underlying enzymes have not yet been identified. Part of these enzymes may accept both NAD and NADP as cofactor and may work either in fixed and/or in unfixed tissues (VINCENT, 1986). Previous experiments have shown (SANDELL, 1985; SAGAR, 1986; VANNEY and YOUNG, 1988; MITROFANIS, 1989) that the suitable cofactor is NADP for retinal neurons. However, recent results obtained on frog retinal tissue (SATO, 1990a), have foreshadowed that conditions, which resulted in superior staining quality in mammalian retinal wholemounts, were not at all optimal for the enzymes present in the anuran retina. Optimising the conditions of NADPHd staining in *Bufo* and *Xenopus* retinal wholemounts can be based on the following considerations:

1) *Use of unfixed tissue.* Fixation appeared to abolish most of the specific staining. Furthermore, the present study showed that the enzyme activity, even in mammalian retinal tissues, is reduced by the application of glutaraldehyde and fully abolished by picric acid.

2) *Pretreatment of retinal tissues with 300 mM sucrose in 0.1 M PB.* Cells, particularly in the osmotically very sensitive unfixed retinae, became slightly dehydrated by this osmotic shock. When the tissues were placed back into the incubating medium, the passive water influx probably helped the substrate and the cofactor molecules to penetrate quickly into the cells.

3) *Use of phosphate buffer.* NADPH reaction is known to be pH dependent. When the pH goes below 6.0 during incubation, the progress of the reaction practically stops. This may happen in unfixed tissues because of the high rate of anaerobic decomposition and accumulation of the acidic intermediates. Since the buffer capacity of the PB is substantially higher than that of the Tris and the tissue culture medium, this buffer keeps the reaction between optimal pH conditions. Other investigators using the SAGAR method on fixed tissue (VANNEY and YOUNG, 1988; MITROFANIS, 1989; SATO, 1990a) also preferred PB as a reaction medium. Manganese ions, because they form insoluble precipitate with the phosphate ions, should not be included in the incubating solution. Therefore, manganese ions are not necessary for the development of the reaction in fixed tissue. The same condition is likely to apply to unfixed tissue.

4) *Improvement of the penetration.* Triton X 100 treatment enhances the speed of reaction. The complete removal of the vitreal tissue from the retinal surface was found to be at least as important as the Triton X 100 treatment. The living vitreal tissue forms a strong penetration barrier that reduces the
speed of the reaction.

Unfixed mammalian retinal wholemounts have been used for tetrazolium studies, however, no specific cell types could be stained with this method (KUWABARA and COGAN, 1960). The use of unfixed tissues in SAGAR’s experiments has failed to give good quality staining similar to the observations of the present study. In contrast, in the anuran retinas specific staining was achieved only by using unfixed tissue.

The different requirements for optimal staining of anuran and mammalian retinal tissues might reflect major differences in the enzymes present in these species. The fixation in the mammalian tissues probably reduced the nonspecific enzyme activity and somehow (i.e., crosslinking certain protein structures) enhanced the activity of certain types of diaphorases located in certain nerve cell types. The observations by SANDELL (1985), that several small cells in the INL of rabbit and in some ganglion cells of cat, monkey and human retina were slightly stained supports this view. In anura, the enzyme activity is almost totally destroyed in fixed tissues (except for photoreceptors). Strong staining of photoreceptor inner segments in fixed tissue has also been reported in the pigeon and frog retinae (SATO, 1990a). The characteristics and the identity of the enzymes underlying this reaction should be examined with biochemical methods.

Although this histochemical reaction does not allow the accurate specification of the reacting enzyme(s), the technique can provide a useful tool for the characterisation of subpopulations of neurons of the anuran retina that contain NADPHd.

REFERENCES


