

Specification of Positional Information in Retinal Ganglion Cells of *Xenopus*: Assays for Analysis of the Unspecified State

(neuronal specificity/retinotectal connections/tissue culture/serial eye transplantation)

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ABSTRACT The central connections of retinal ganglion cells are retinotopically organized, producing a "map" of the retina on the surface of the optic tectum. Exactly how and when individual ganglion cells develop the position-dependent properties (termed *locus specificities*) subserving formation of the map is unknown, but the positional information that each ganglion cell will use in this process is specified in the early *Xenopus* embryo during a critical period at stages 28-32. We report two methods for isolating eye primordia from the axial cues of the animals during this critical period and for then allowing the eyes to form retinotectal connections in a carrier embryo. The results show that, as early as optic vesicle stages 22-23, the eyes already contain orthogonal reference axes, that positional information can be specified with respect to these axes *in vitro*, and that the specification process itself may only entail a transition from a reversible to an irreversible state.

Neuronal *locus specificity* in retinal ganglion cells is defined as the property of the individual cell that predisposes its axon to synapse at a particular locus in the retinotectal map (1). All available evidence indicates that locus specificity is a surface-active cytochemical property, which is correlated in the normal animal with the cell's position in the retina (2, 3). Exactly when the individual ganglion cells develop definitive locus specificities is unknown and may differ for different ganglion cells. Nevertheless, the developmental program for spatial organization of the *entire set* of retinal locus specificities appears to be set down in the early *Xenopus* embryo (before optic nerve development) during a 5- to 10- hr "critical period" at stages 28-32 (4, 5).

Recently we showed (3) that locus specificity appears to derive from field *positional information* (6), that is, information about the cell's position *in the retina*, as measured along two orthogonal reference axes (anteroposterior and dorsoventral) that are established as the result of interaction with the general axial cues of the embryo. The programming events at stages 28-32 effect the "locking-in" of such a pair of reference axes, accompanied in the intact eye primordium by the determination, or *specification*, of the positional information that all the ganglion cells will use to develop appropriate locus specificities. A change of state *within the eye* itself was directly demonstrated and operationally characterized in intact stage 31/32 eyes as stable and irreversible,

Abbreviation: VGSRs, visually guided strike responses.

This is paper III on "Specification of positional information in retinal ganglion cells of *Xenopus*." Paper II is ref. 1.

rendering the retinal cells refractory to information about changes in their positions thereafter (1). Thus, completely inverted retinotectal maps developed when stage 31/32 eyes were grafted in 180° rotated position into host orbits, even when the host had not yet reached the critical period, or when the eye was cultured *in vitro* for 6-10 days before reimplantation.

These experiments provided no insights, however, into the nature of the "unspecified state" in eyes before stage 28. In fact, conventional orbit-to-orbit transfer experiments on unspecified eyes have consistently failed to provide such insights, for these eyes go on to interact with the axial cues of the host embryo, specify positional information with respect to the host-derived retinal axes, and ultimately generate normal retinotectal maps. Here we present two methods in which unspecified right eyes are (i) removed from *donor* embryos before stage 28, (ii) cultured during the critical period in isolation from all axial cues (either *in vitro* or on an *intermediate host* at a site where axial cues do not influence the eye), and (iii) reimplanted into the enucleated right orbits of *final carrier* embryos, where retinotectal connections are allowed to develop and are assayed when the carrier reaches metamorphosis. These methods have permitted a preliminary characterization of the unspecified state in eyes before stage 28, and they offer the possibility of determination of the temporal parameters of important programming events in normal and transplanted eyes.

MATERIALS AND METHODS

The experiments encompassed many variations of embryonic stages and durations of culture. We will describe two exemplary series in detail and allude more briefly to the variations in the terminal section of this article.

Series I (Eye Development on Ventral Midline). Stage 25/26 embryos were collected, dejellied, and anesthetized in 1:10,000 MS-222 (Sandoz). A wound was prepared on such an embryo (the intermediate host) on the ventral midline, anterior to the anus but well posterior to the heart rudiment. The right eye primordium was surgically excised from another stage 25/26 (donor), with overlying ectoderm intact, and implanted in a specific orientation (see below) into the host's wound. Hosts developed under close observation for 3-4 days in 15% saline, reaching early 40s stages; surgical failures were discarded. Remaining hosts were again anesthetized, along with sibling stage 39/40 embryos (final carriers). The right

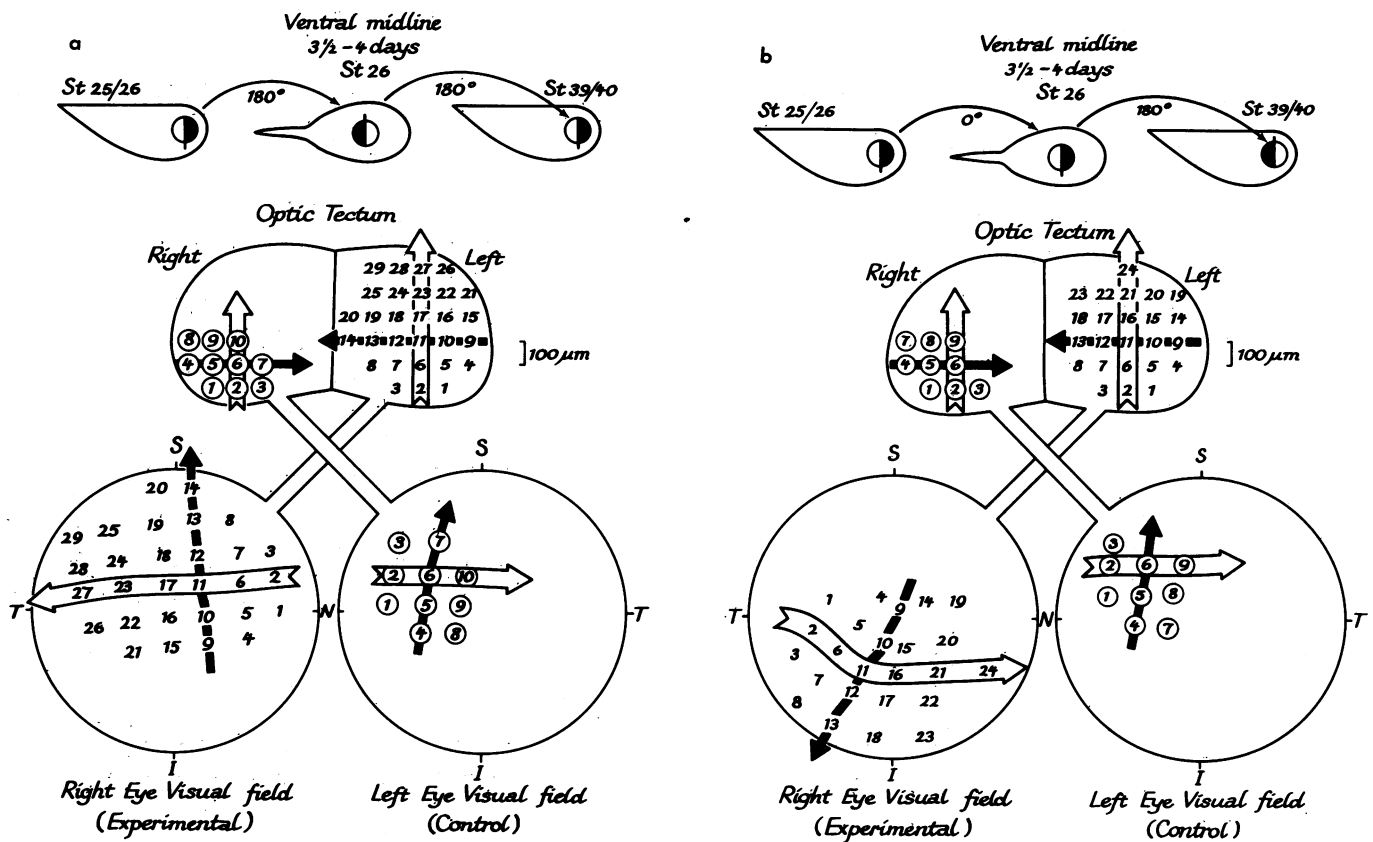


FIG. 1. (a) Diagrammatic summary of Type Ia procedure (top), showing the orientation of the eye on the donor, intermediate host, and final carrier (anatomical anterior half of the eye is shaded and a key projects ventrally), and the visual field projections onto the tectum in a typical type IA carrier (bottom). The right eye of the frog was centered, which permits complete mapping of the visual field projection of the experimental right eye onto the left tectum, but limits mapping of the right tectum as described (1). Each number in the visual field shows the position of the stimulus that optimally evoked potentials recorded by an electrode at the position shown by the same number on the contralateral tectum. The distance between tectal electrode positions is shown by the bar, and the visual field extends 100° from center to periphery. Conventions are the same for other figures. (b) Type IB procedure and the visual field projections of a typical Type IB carrier. (c) Type IC procedure and the visual field projections of a typical Type IC carrier.

eye of the carrier was removed; the experimental eye was excised from the host belly and placed in a specific orientation in the carrier orbit. In Type IA, the eye was inverted anteroposteriorly during both transfers, thus ending up in normal anatomical orientation in the carrier orbit; the remaining eyes were finally rotated 180° in the final carrier orbit, having been inverted anteroposteriorly only during the second transfer (Type IB) or only during the first transfer (Type IC).

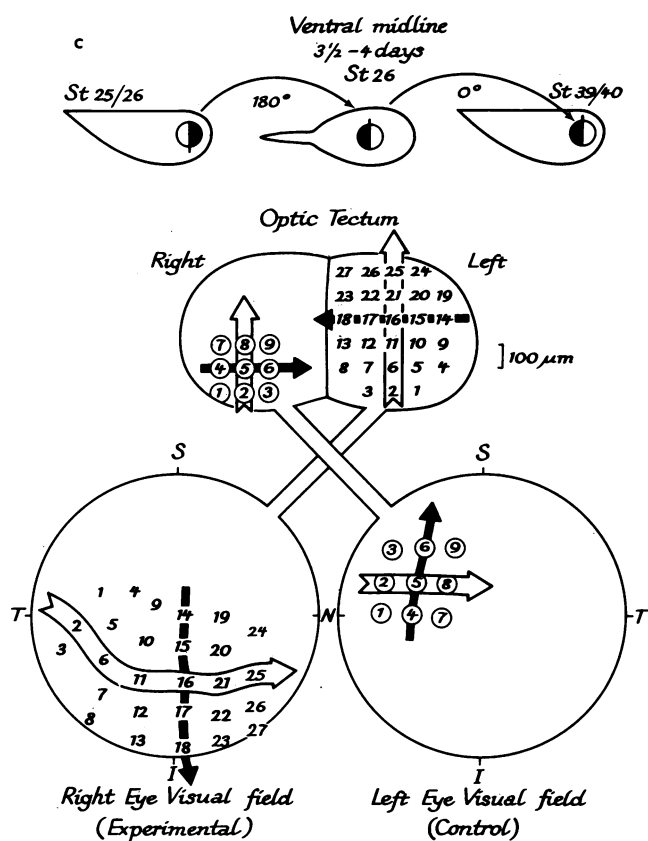
Series II (Eye Development In Vitro). Stage 22 embryos were disinfected by 5-sec immersion in 70% ethanol, rinsed, and dejellied aseptically in culture medium [Steinberg's saline (8) with 100 U/ml of penicillin, 0.25 $\mu\text{g}/\text{ml}$ of Fungizone, 100 $\mu\text{g}/\text{ml}$ of streptomycin]. Right eyes were removed with overlying ectoderm intact from unanesthetized donors, transferred in groups of three to four into a 10-ml petri dish containing 4–5 ml of medium, and cultured at room temperature for 3–4 days. Sufficiently "mature explants" (see below) were then reimplanted into the enucleated right orbits of stage 39/40 sibling carriers. Eyes were reimplanted in normal (Type IIA) 180° -rotated (Type IIB), or 90° -rotated (Type IIC) orientations.

The explants were oriented randomly in the dish; they

shifted their orientations whenever the cultures were moved for examination. The amount of orbital tissue included in the explant was negligible (indeed, in many cases the fringes of the eye were left in the donor orbit) as judged by visual inspection at $\times 50$ magnification and from histological sections of explants fixed during the culture period.

Development of Cultured Eyes. After a short lag, the Series I eyes developed synchronously with the host's own eyes. In 3–4 days, they showed the histological and morphological characteristics of larval eyes (early 40s stages), including bushy optic nerve fibers that ramified in the subcutaneous tissue. In contrast, the eyes cultured *in vitro* varied in rate and extent of development. All the "mature explants" in the Series II experiments, however, showed the morphological and histological characteristics of stage 37/38 eyes (e.g., clear cornea, smooth black choroid, retinal layering, and extensive optic nerve growth), completely encapsulated in a transparent epithelial pouch. A detailed analysis of the origin and cytodifferentiation of various neuronal phenotypes in these cultured eyes will be published (Hunt, R. K., Bergey, G. & Holtzer, H., in preparation).

Retinotectal Mapping. Published procedures (1, 3) were used for (i) rearing carriers through metamorphosis, (ii) testing



their visually guided strike responses (VGSRs), and (iii) electrophysiologic mapping of the projection of each eye's visual field onto the contralateral optic tectum. The last procedure above (in which a platinum-iridium microelectrode is moved across the tectum in 100-nm steps and, at each electrode position, the optimal stimulus position in the visual field is determined) assays for some parameters, such as the directions of the axes, of the set of specificities in the eye. Although not illustrated, recordings were made from intertectal visual fibers (9) to confirm the existence of functional visual synapses from the experimental eye. Maps from the carrier's normal left eyes are shown but not discussed. The optic nerve from the experimental eye failed to reach the tectum in 10% of Series I carriers and 50% of Series II carriers, and the *Results* derive from the remaining 33 carriers.

RESULTS AND DISCUSSION

Controls. To confirm that the experimental eyes were initially unspecified, we used control stage 22 or 25/26 embryos (siblings of, and reared with, the experimental donors). Right eyes were (i) removed and replaced without any disorientation, or (ii) rotated 180° *in situ*, or (iii) transplanted, with rotation, into the enucleated right orbits of the Series I or II donors. Less than half these eyes projected to the tectum, but in those the retinotectal maps were all normal (i.e., appropriate to the position of the eye after surgery).

Series I (Eye Development on Ventral Midline). One Type IA carrier showed disorganized retinotectal projection, the reasons for which are uncertain. All eight others, however, showed normal VGSRs and normal retinotectal maps (Fig.

1a). These normal maps indicate that Series I procedures do not inherently alter the pattern of connections that develops from the experimental eye, and that such axial cues as may be present at the intermediate host culture site played no role in organizing the set of locus specificities that ultimately developed. In the Type IB and IC experiments, in which the eye was inverted anteroposteriorly only *once* and terminated in 180°-rotated orientation in the carrier, all carriers showed misdirected VGSRs and complete inversion of the retinotectal map from the experimental eye (Figs. 1b and c). This result means that the embryonic axial cues of the carrier also played no role in organizing the set of locus specificities in the eye. Thus, in the Series I eyes, the reference axes for positional information in the retina were acquired in the donor embryo, before surgical intervention at stages 25/26.

Series II (Eye Development *In Vitro*). All carriers with the eye in normal orientation (Type IIA) showed normal VGSRs and normal retinotectal maps (Fig. 2a), indicating that the *in vitro* procedures do not in themselves alter the eye's ability to generate a normal pattern of retinotectal connections. All Type IIB carriers, with 180°-rotated eye, showed misdirected VGSRs and completely inverted retinotectal maps (Fig. 2b). Finally, most Type IIC carriers, where the eye was in 90°-rotated orientation in the carrier orbit, also showed misdirected VGSRs; and their retinotectal map from the experimental eye was rotated 90° from normal (Fig. 2c).

In several Type IIC carriers, the eye had physically "derotated" to assume its normal anatomical orientation in carrier orbit, and the retinotectal map from the experimental eye was completely normal. This phenomenon, previously described by Harrison in transplanted limb buds (10), is currently being investigated.

Thus, the results from Type IIB and IIC experiments indicate that the reference axes for positional information were acquired in the donor embryo, *before stage 22*, and that 180° disorientation of the eye at the time of reimplantation is not necessary for positional information to be specified along these previously-acquired reference axes.

These experiments permit several basic conclusions. First, the eye primordium before stage 28 is not spatially undifferentiated or without markers reflecting its orientation on the animal. In fact, the *stage 22* eye primordium already possesses directional differences ("polarities") along two orthogonal axes. We make no assumptions about their cellular basis or precise localization; they may be identical to the reference axes in specified eyes, or they may reflect more primitive directional markers that can generate such reference axes later. Nevertheless, these primitive axes are aligned with the anteroposterior and dorsoventral axes of the donor embryo, and they can serve as (or transform into) the reference axes for positional information, specified at a later time.

Second, it follows that the programming events at stages 28–32 do not impart polarity to the eye or involve any obligatory interaction between the eye and the embryo's axial cues. Indeed, that a spatially organized set of locus specificities developed at all in the Series II eyes is remarkable, for these eyes never experienced the embryonic milieu while they were *in vitro* from stages 23–38. Apparently, then, there is *nothing* unique to the embryo during the critical period at stages 28–32 that is indispensable to the emergence of reference axes in the retina or the specification of positional information for the ganglion cells.

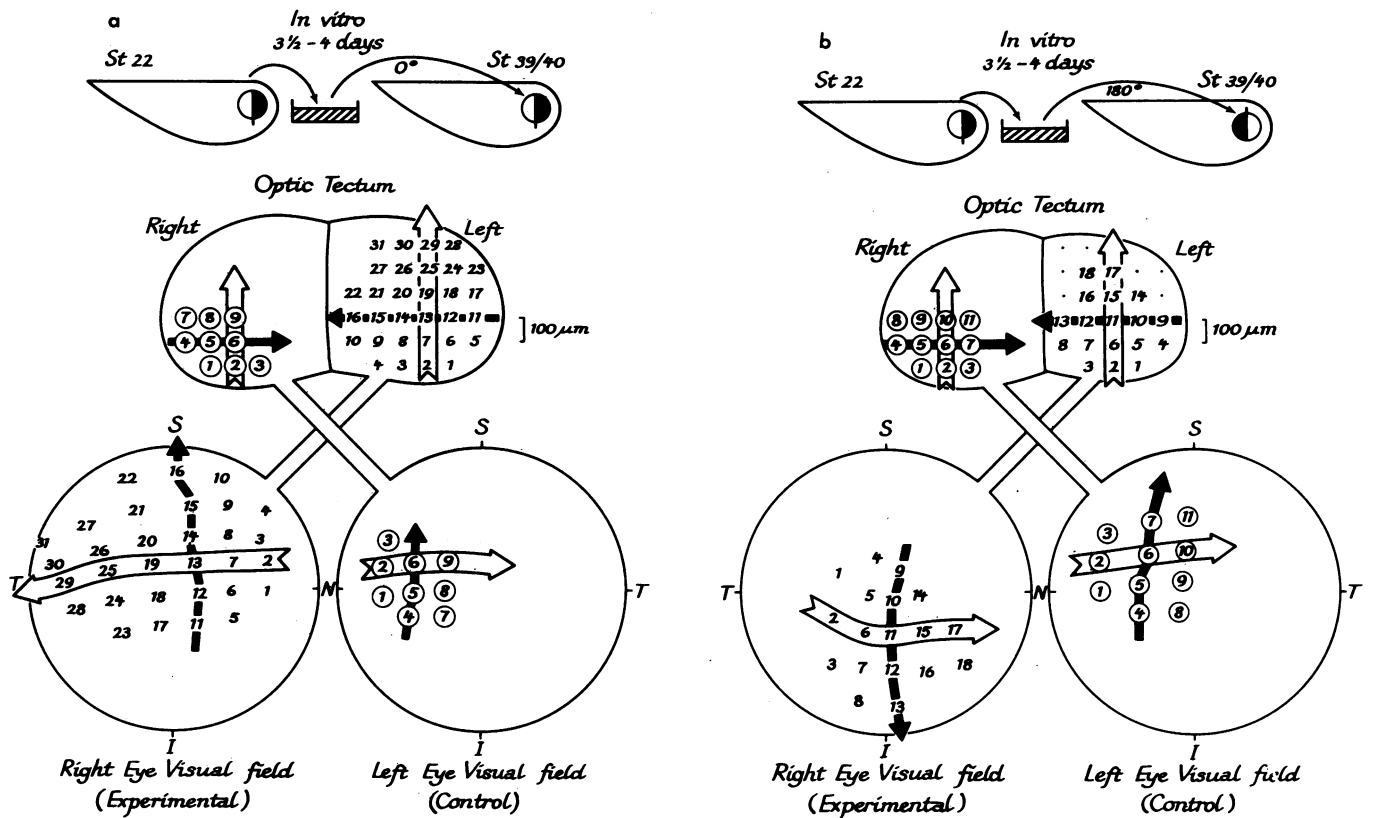


FIG. 2. (a) Type IIA procedure and the visual field projections of a typical Type IIA carrier. (b) Type IIB procedure and the visual field projections of a typical Type IIB carrier. (c) Type IIC procedure and the visual field projections of a typical Type IIC carrier.

Third, while the unspecified state is, by definition, reversible—the primitive axes are replaced by new axes when the unspecified eye is reoriented in the orbit (ref. 5 and controls above)—it is also a stable state, for the primitive axes survived on the intermediate host in the absence of further interactions between eye and axial cues, as well as *in vitro* in total isolation from the embryo.

Variations and Applications. The results described above have now been confirmed for unspecified eyes at several stages and for stage 30 eyes, in which anteroposterior specification has occurred but dorsoventral specification has not (1, 5). Moreover, when a *left* eye is cultured as in Series II and reimplanted in normal dorsoventral orientation in the *right* orbit of a stage 39/40 carrier, the eye generates the set of

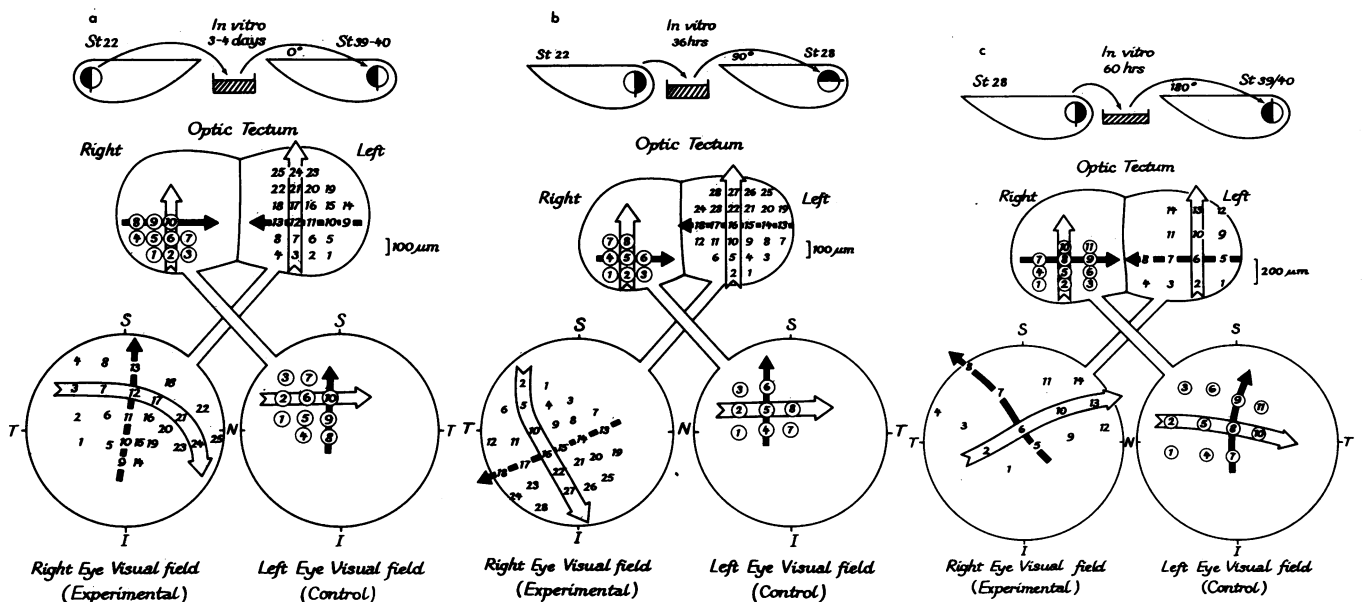
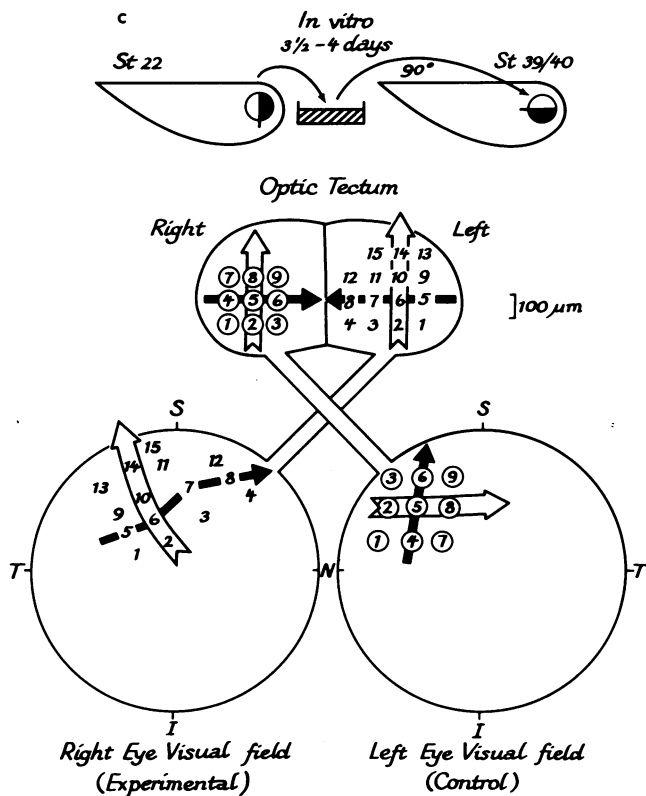


FIG. 3. Visual projection of carriers in experiments similar to Series II, but modified as described in the text. Modified procedures are sketched above the respective visual projection diagrams in Fig. 3a, b, and c.



locus specificities appropriate to a left eye and maps into the tectum with one axis inverted (Fig. 3a). Additional experiments have shown that the programming events that specify positional information can occur when the eye is *in vitro*, and that the specified state in such an eye is just as irreversible as that in a normal stage 31/32 eye. Series II procedures were modified to use stage 28 carrier embryos, which possess all the conditions necessary to establish a new pair of reference axes in the eye (5). The retinotectal maps in these carriers showed the same correlation with the final orientation of the eye that was observed in Series II above (Fig. 2a-c). Moreover, when progressively shorter culture periods were used, *in vitro* specification of both the anteroposterior and dorsoventral axial components of positional information can occur in less than 36 hr (Fig. 3b).

Studies similar to Series II but involving the reimplantation of "less mature" explants (eyes before stage 28 which *in vitro* attained the morphological and histological appearance of eyes at stages 30 to 34) have not yielded consistent results. Although the reasons for this variation have not been identified, all the eyes reimplanted in normal orientation in the carrier formed normal retinotectal maps, indicating that the variability is not due to random axial changes in the eye. Thus, when reimplanted in 180°-rotated position, these "less mature" explants usually developed completely inverted retinotectal maps, but in four cases (after 60–70 hr *in vitro*) the resulting retinotectal map was inverted anteroposteriorly, but normal in the dorsoventral axis of the eye (Fig. 3c).

These results are significant in two respects. First, several of the eyes that ultimately generated completely inverted retinotectal maps had lost their overlying ectoderm after explantation and had developed stark naked *in vitro*. This result completely eliminates the notion that adherent periorbular tissue is "polarizing the eye" *in vitro*. Second, the retinotectal maps inverted anteroposteriorly after such a procedure indicate that at least the dorsoventral-axial cues are still present at stage 39/40, and positional information can be specified along two reference axes established 60 hr apart in the eye.

The results in this paper, then, are primarily illustrative of an approach, whose potential remains to be fully realized. Some uncertainties in the assay have yet to be clarified: (i) whether the anteroposterior cues are present in stage 39/40 carriers; (ii) why, and whether under all conditions, the eyes fail to show the influence of such cues on the ventral midline; and (iii) whether the acquisition of a new, carrier-derived dorsoventral axis in some of the "less mature" explants resulted from the loss of the eye's primitive dorsoventral axis *in vitro*, or from the failure of dorsoventral specification to occur. Nevertheless, these assays can be modified to provide answers to these questions, to obtain basic information about the time required for axial replacement and for the unspecified-to-specified state transition, and to allow selective interference with these processes by various pharmacological agents and elucidation of their cellular basis.

For the present, however, we may conclude that the unspecified state in the *Xenopus* eye before stage 28 is a stable but reversible state, which includes a set of primitive axial markers that are aligned with the orthogonal body axes of the embryo. Whether these primitive axes are simply a modifiable version of the definitive reference axes characteristic of the specified state remains to be determined. But the eye can "lock-in" on these primitive axes and specify positional information for an entire, spatially-organized set of locus specificities in the absence of any further interactions with the embryo.

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