

The Development of Anucleolate Embryos of *Xenopus laevis*

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WITH PLATE

A MUTATION of *Xenopus* has been found which reduces the maximum number of nucleoli per diploid nucleus from 2 in the wild-type to 1 in heterozygotes. Homozygous mutants possess no true nucleoli, hence being termed *anucleolate*. They do, however, possess pyronine-staining intranuclear organelles that are smaller and more numerous than typical nucleoli. The mutation can be considered as a recessive larval lethal, unlinked to sex: heterozygotes of both sexes are fully viable, but anucleolate larvae die at about the time their sibs begin to feed. These points have been recorded in two preliminary reports (Elsdale, Fischberg & Smith, 1958; Fischberg & Wallace, 1960). The development of anucleolate embryos is described here as a basis for the design of an experimental analysis of the ways in which the mutation acts. The description rests mainly on the progeny of a single mating of heterozygous toads, conforming with notes made on other such matings, from which additional data are drawn when required. As no developmental differences have been detected between the wild-type and heterozygotes, they are treated together as nucleolate (+*n*) controls, to which the anucleolate (*On*) embryos are compared. Nieuwkoop & Faber's (1956) stages of normal development are used here to indicate age, made independent of temperature. After stage 40, the retarded *On* larvae are assigned to the stage of their controls.

EXTERNAL DESCRIPTION

Camera lucida drawings and records of some physiological and morphological characters have been made on a random sample of embryos from stage 28 onwards. The characters studied are summarized in Table 1. Successive drawings of a typical *On* tadpole and a +*n* control are shown in Text-fig. 1.

Anucleolate embryos hatch during the same period as controls (stage 35/36). No differential mortality occurs before this. Soon after hatching, control larvae swim upwards and suspend themselves, by means of the cement gland secretion, from the water surface or the sides of the container. *On* larvae are relatively

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inactive in this respect (Table 2). This difference plays an important part in the design of suitable culture conditions: *On* larvae must be isolated after hatching. Otherwise they are rapidly infected by the decaying eggs found at the bottom of most mass cultures.

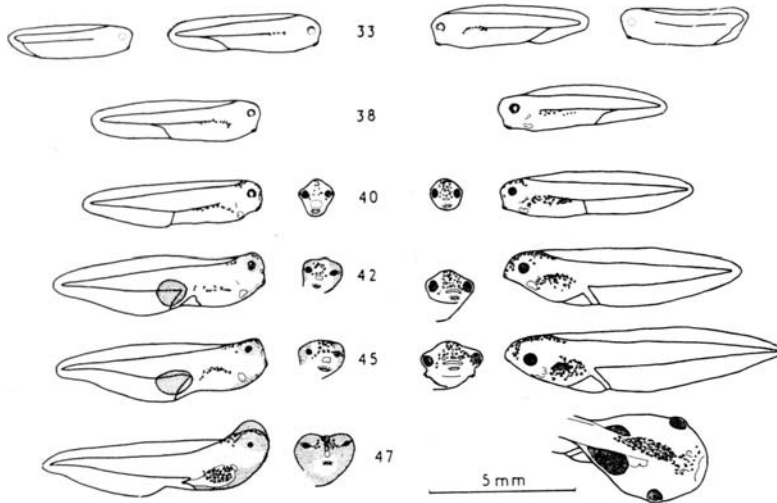


FIG. 1. Successive drawings of a typical *On* larva (on left) and a control larva (on right). The numbers refer to stages of normal development. Cranial and trunk melanophores and the retinal pigment are shown in black; other organs in outline only. Oedemata are stippled, and they are encircled where protruding from the body contour.

TABLE 2
Suspension of newly hatched larvae

	Controls	Anucleolate
Became suspended in 3½ hours	579	38
Remained at bottom of tank for 3½ hours	91	173
TOTAL	670	211

A sample of newly hatched larvae were shaken to the bottom of a tank containing 2 inches of water. Those that rose and became suspended were collected periodically, and diagnosed at stage 40. The heterogeneity ($\chi^2_{(1)} = 354.6$; $P < 0.001$) shows that anucleolate larvae are relatively inactive. The proportion of anucleolate larvae in the sample agrees with a 3:1 ratio ($\chi^2_{(1)} = 0.412$; $P > 0.5$); no differential mortality has occurred.

Anucleolate larvae are externally indistinguishable from their sibs up to stage 39. After this, the jaw-growth of control larvae pushes the cement gland to a high anterior position (Text-fig. 1). The cement gland of *On* larvae remains in a ventral position, very slightly in front of the eyes. This is the earliest distinguishing morphological character found. Assuming good culture conditions and the absence of microcephalic controls, it is quite reliable by stage 40. After stage 40, *On* larvae become noticeably retarded in such aspects of growth as

tail-expansion, head-enlargement, gut-coiling, heart-growth, and the spreading of melanophores over the head and flank. As *On* larvae show few signs of life (the heart-beat may escape detection), they were considered dead only when they had decayed so far that the oedemata had collapsed and their eyes had swollen (Table 1; relative survival). Some might have survived longer in more sterile culture but they were obviously incapable of feeding. The survival of all the more active unfed sibs shows that starvation was no cause of death.

Two observations in Table 1 require comment. The blood circulation of *On* larvae at first seems completely normal and can even be traced through the rudimentary external gills. Later, the blood corpuscles settle in such areas as the pronephric sinus and subcaudal vein. Corpuscular movement was very rarely observed after stage 44, although the heart continues to beat. This may probably be attributed to the oedematous condition of the *On* larvae and to an inferior pumping action of the heart. When motionless stage 40 tadpoles were compared, *On* larvae showed a subnormal rate of heart-beat. The mean time of 100 beats recorded at 23–24° C. was 55.2 ± 0.639 seconds for 20 control larvae, and 71.0 ± 1.395 seconds for 20 *On* larvae. A *t*-test shows this difference to be significant ($t = 11.356$ for 38 degrees of freedom, $P < 0.001$). The cement gland normally ceases to secrete at about stage 46 and is degenerating by stage 47. The expected degeneration of the cement gland of *On* larvae is delayed; they can be attached to the water surface until shortly before death.

Elsdale *et al.* (1958) mention, as characteristic of *On* larvae, that the tail-tip is crumpled and bent ventrally. I suspect this to be an effect of suboptimal culture conditions, but there is a tendency for *On* larvae to show this character. From three samples of stage 43 *On* larvae (from successive matings) 7 per cent., 20 per cent., and 48 per cent. showed a ventral flexure of the tail-tip.

MICROSCOPIC EXAMINATION

Groups of embryos were fixed in Zenker at various times corresponding to normal stages of development of the controls (with *On* embryos of the same age). They were cut in 8μ transverse sections and stained with Jordan & Baker's (1955) mixture of pyronine and methyl green. The description below is based upon the following numbers of *On* embryos: 1 at stage 28, 2 at stage 29/30, 4 at each of stages 31, 35/36, 39, 40, 42, 45, 47; and an equal number of controls at each stage.

Central nervous system

The brain of *On* embryos is normal in shape and size up to stage 31, but already contains an excessive number of cells with pycnotic nuclei (Text-fig. 2). These fall into the lumen of the brain, where cell debris is found between stages 35/36 and 42. By stage 42 the brain has partially collapsed and the organization of nuclei and axons is irregular. Most of the pycnotic cells have been lost by stage 45. No further significant pycnosis occurs until death. Mitosis occurs at all

stages in the cells lining the lumen of the brain, but is less common in the later stages of *On* larvae (Text-fig. 2). This is partly due to the fact that a proliferation of the mid-brain floor occurs at stages 40, 42, and 45 only in controls. The spinal cord of *On* larvae shows the same syndrome as the brain, although delayed in its caudal region. Pycnotic cells appear and are discharged into the lumen of the cord. The formation of both dorsal and ventral nerve roots is delayed. The

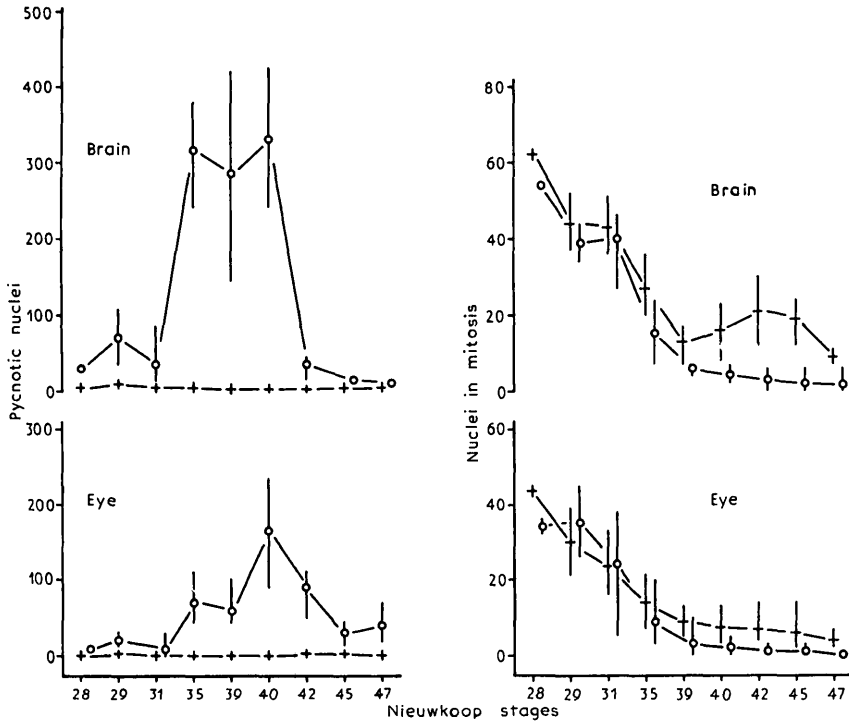


FIG. 2. Counts of pycnotic nuclei and mitotic figures, made on 3 adjacent sections of the midbrains and eye-cups. The mean values and ranges of the counts are shown against the developmental stage. *o* — *o*, anucleolate larvae; + — +, control larvae.

auditory ganglion possesses some pycnotic cells in its early stages. The primary optic vesicle is of normal shape and size, and invaginates normally (stages 31–35/36). It is also subject to pycnosis, cell debris being discharged into the cavity between the eye-cup and the lens. The retina collapses on to the lens at about stage 39. Differentiation of the retinal layers proceeds slowly and is disturbed by patches of pycnotic cells. Counts of mitotic figures and of pycnotic nuclei in the eye-cup are given in Text-fig. 2.

Other ectodermal derivatives

The ectoderm is competent to form nasal and auditory placodes and the lens. The epidermis contains the normal large unicellular glands after stage 40: apart from the wrinkles caused by subsided oedemata, it seems to be normal. The

cement gland has a perfectly normal structure and is retained throughout life. The lens is initially normal, forming a mass of primary fibres (stage 35/36). There is little or no secondary growth of the lens by addition of fibres from the retinal side of the lens. Also, the lens is permanently attached to the overlying epidermis—which is only a transient phase of normal lens development. Pycnotic nuclei have been observed only occasionally in this tissue. The auditory vesicle and nasal placode both contain pycnotic cells at stages 28–42. The development of both is retarded. The visceral arch ectomesenchyme appears as a rather looser tissue than in controls. Both the jaw cartilage and transverse mandibular muscle anlagen contain pycnotic cells (stages 35/36–42). After stage 39 the condensation of these anlagen is distinctly retarded; some differentiation into muscle fibres and cartilage is followed ultimately by the formation of some cartilage in the gill-arches. These ectomesenchymal structures are inferior to those developed by controls at stage 42.

Mesoderm

The development of the trunk notochord and somites is identical to that of controls. The heart has a normal appearance until stage 40 but remains a simple twisted tube, while the heart of *+n* larvae develops a thicker myocardium, ventricular trabeculae (stage 42), and two auricles by stage 47. The *On* heart contains no pycnotic tissue. The pronephros develops normally up to stage 39, when it has three ciliated funnels, a slightly coiled collecting tube, and a duct opening to the cloaca. Little further coiling of the collecting tube occurs, and it is often swollen into a series of bladders. By stage 40 the pronephric sinus is gorged with erythrocytes and cell debris. The latter is found within the lumen of the pronephros and its duct. Presumably the cell debris comes from other tissues and is being excreted: the pronephric tissue itself appears to be perfectly healthy. No divergence from normality has been found in the lateral plate mesoderm.

Endoderm

The resorption of the postanal gut is not markedly delayed, but intestinal coiling is retarded; only a single gut loop is achieved, corresponding to that of stage 42–43 controls. The mouth opens at the usual time (stage 40). The cells of the pharyngeal floor of control larvae proliferate (stages 35/36–40) to form a medial area of subepithelial columnar cells, which constitute the bulk of the 'primitive tongue', and three gill-strands on either side. A similar proliferation is found at the same time in *On* larvae but is accompanied by pycnosis, beginning at stage 35/36 and judged to be most intense at stage 42. No columnar cells are formed, and the break-through of the gills is delayed. The larynx and lung diverticula appear late and grow slowly. The liver eventually develops lacunae containing erythrocytes and some pycnotic cells, which may be found in all parts of the blood-stream and so are not particularly associated with the liver.

The various parts of the gut can be recognized by their position, with the exception of the pancreas, which has not been identified. The stomach, duodenum, and intestine show little differentiation, and a moderate amount of pycnosis that is not strikingly greater than may be encountered in control tadpoles.

DISCUSSION

These observations lead to the conclusion that *On* embryos develop at a normal rate until about the time of hatching, after which they become increasingly retarded and abnormal. Their development is not completely arrested, at least until they correspond in appearance to stage 42 controls.

The incidence of pycnosis suggests that it is related to cellular differentiation. In the midbrain and eye-cup, pycnosis reaches its maximum intensity between stages 35/36 and 40 (Text-fig. 2). The majority of cells survive this period and are able to differentiate. Thus the pycnosis is quite distinct from that which affects moribund tadpoles. There are two observations which support the contention that this neural pycnosis is not correlated with abnormal cell-division. Firstly, all phases of mitosis are seen and appear completely normal. Secondly, at the stages considered, cell-division is virtually restricted to cells lining the lumen of the brain and spinal cord (or its vestige in the eye-cup), yet pycnosis is found in all parts of the neural tissues. The rather low mitotic counts of *On* brain and eye-cup (Text-fig. 2) may result from the reduced number of cells, or from a lower rate of mitosis that might be expected from the generally retarded development.

Although it is difficult to distinguish between primary and secondary abnormalities on the basis of a purely descriptive study, it seems clear that the abnormality of the central nervous system is caused neither by defective induction nor by a deficient circulation. The central nervous system of tail-bud stages has a normal conformation (unlike that of typical microcephalic larvae), while neural pycnosis is common before stage 33/34 when the heart normally begins to beat. The defective lens development may be dependent on the abnormal eye-cup, as has been demonstrated to be the case in several amphibians (Balinsky, 1957). The pharyngeal pycnosis and atypical differentiation could also be a secondary abnormality; Okada (1957) has shown that the pharyngeal differentiation of urodeles is induced by ectomesenchyme. The retarded development and growth of other organ systems may result from the inferior blood circulation.

SUMMARY

1. Anucleolate *Xenopus* embryos develop into normal neurulae and tail-bud stages. After hatching, the development of all their organs is retarded. Only then can anucleolate larvae be recognized by such external characters as relatively small brains, eyes and jaws, and anal (followed by other) oedemata.

2. An abnormally large number of pycnotic nuclei occur in the central

nervous system, eye-cup, auditory vesicle, nasal placode, and visceral arch ectomesenchyme. The majority of cells of these tissues are not affected by pycnosis, and are able to differentiate.

3. The mesodermal organs are not affected by pycnosis. Their development is retarded after hatching.

4. Of the endodermal organs, severe pycnosis is encountered only in the pharyngeal floor, which differentiates atypically.

5. It is suggested that the primary abnormality of these mutants concerns the central nervous system, neural crest, and sensory ectodermal derivatives. The abnormal development of the lens and pharynx may be caused by abnormal secondary inductions.

RÉSUMÉ

Le développement des embryons anucléolés de Xenopus laevis

1. Les embryons anucléolés de *Xenopus* se développent normalement jusqu'au stade de la neurula et du bourgeon caudal. Après l'éclosion, le développement de tous les organes est retardé. C'est alors seulement qu'il est possible de reconnaître les larves anucléolées à la taille relativement petite de leur cerveau, de leurs yeux, et de leurs mâchoires, et à la présence d'oedèmes anaux (bientôt accompagnés d'autres oedèmes).

2. On trouve un nombre anormalement grand de noyaux pycnotiques dans le système nerveux central, la cupule optique, la vésicule auditive, les placodes nasales, et l'ectomesenchyme des arcs viscéraux. La majorité des cellules de ces tissus n'est pas touchée par la pycnose et se différencie normalement.

3. Les organes mésodermiques ne sont pas affectés par la pycnose. Leur développement est retardé après l'éclosion.

4. Seul, parmi les organes endodermiques, le plancher pharyngien est sévèrement atteint par la pycnose. Sa différenciation est atypique.

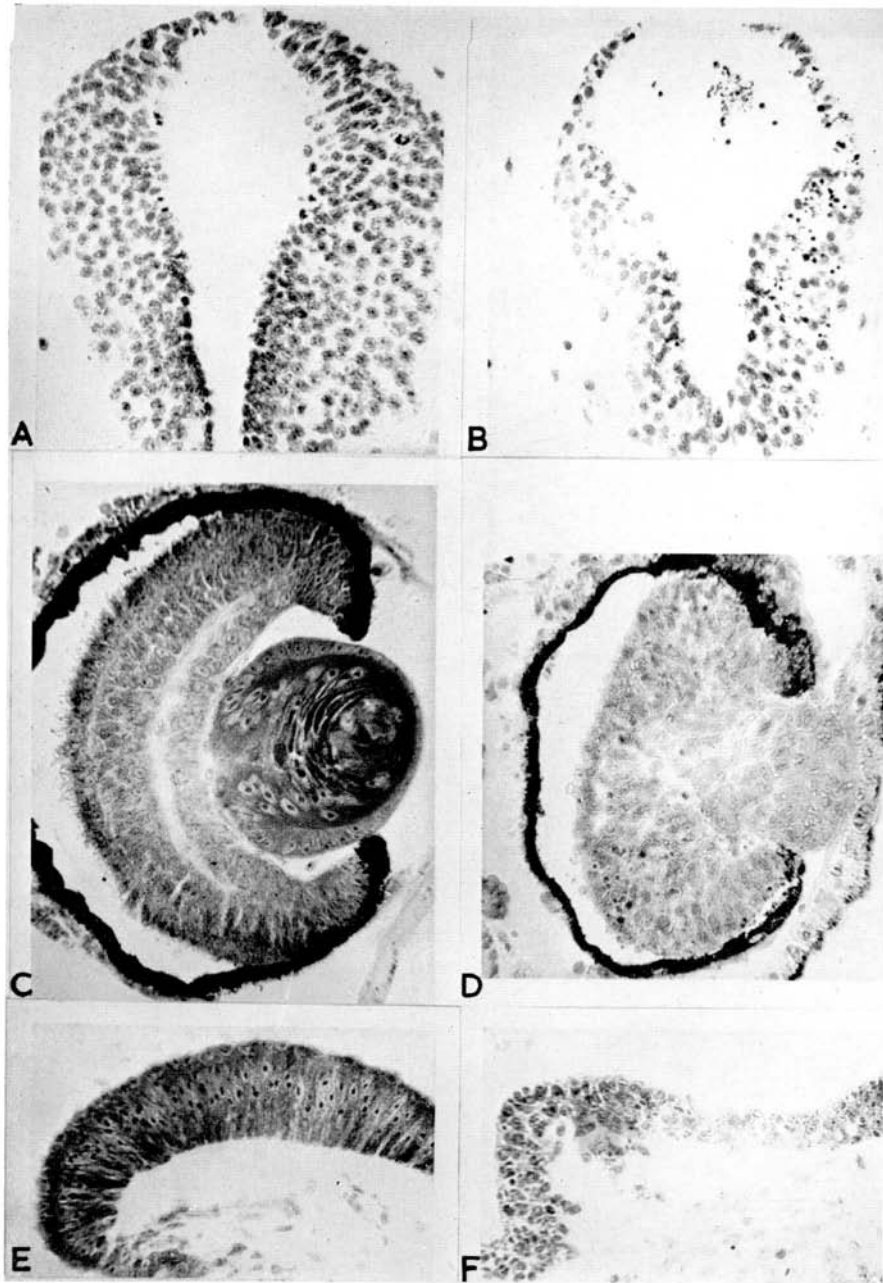
5. Il semble que les anomalies primaires de ces mutants affectent le système nerveux central, la crête neurale, et les organes des sens d'origine ectodermique. Il est possible que le développement anormal du cristallin et du pharynx soit dû à des inductions secondaires anormales.

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EXPLANATION OF PLATE

- FIG. A. Midbrain of stage 40 control larva, showing cell-division but no pycnosis.
- FIG. B. Midbrain of stage 40 *On* larva, showing pycnotic nuclei, cell debris, and one metaphase.
- FIG. C. Eye and lens of stage 40 control larva, showing one pycnotic retinal cell.
- FIG. D. Eye and lens of stage 40 *On* larva, showing retinal pycnosis and the poor development of both tissues.
- FIG. E. Pharyngeal floor of stage 47 control larva, showing columnar cells of the 'primitive tongue'.
- FIG. F. Pharyngeal floor of stage 47 *On* larva, showing the atypical cellular differentiation.
- (FIGS. A, B—feulgen stain, green filter; dark nuclei. FIGS. C, D, E, F—pyronine-methyl green stain, green filter; dark nucleoli.)

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