Studies on the effect of monoamine antagonists on the morphogenesis of the newt

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ABSTRACT. The effects of three monoamine antagonists, p-chlorophenylalanine, diethyldithiocarbamate and propranolol on the morphogenesis of newt embryos were studied. Antagonists were administered during late blastula through neurula stages. In a concentration of 1 mM, all three arrested gastrulation and caused disintegration of the embryos. Lower concentrations (0.1-0.5 mM) retarded morphogenetic movements in the gastrulation and caused malformations especially in the anterior parts of the embryos; pigmentation was delayed by 1 or 2 days. In addition, p-CIPhe inhibited yolk granule degradation in the notochord and DEDTC caused notochordal hypertrophy.

The results show that interference with synthesis or action of catecholamines and serotonin affects morphogenesis. With the methods used it is not possible to discover exactly how monoamines regulate the morphogenetic events because of the unspecific side effects of the antagonists and the feedback interactions between the monoamines.

KEY WORDS: *monoamine antagonists, monoamines, amphibian morphogenesis*

Introduction

Monoamines, which act as chemical mediators or neurotransmitters in nervous systems, appear in animal embryos before any sign of neural differentiation (see Lauder, 1983). They initiate and regulate morphogenetic movements in echinodermates (Gustafson and Toneby, 1970, 1971). Monoamines have also been found in vertebrate embryos during the active phases of morphogenetic movements and formation of organ primordia; catecholamines, for instance, occur in the neurulae and tailbud embryos of the toad (Miranda *et al.*, 1982) and serotonin is present during gastrulation and neurulation processes in the chick embryo (Wallace, 1982; Emanuelsson *et al.*, 1988). However, their functions in the early vertebrate embryos are less well known. The object of our study was to investigate the possible role of monoamines in amphibian morphogenesis using monoamine antagonists as experimental tools.

Results and Discussion

Two of the monoamine antagonists used, p-chlorophenylalanine (CIPhe) and diethyldithiocarbamate (DEDTC) are enzyme inhibitors. CIPhe inhibits the activity of tryptophan hydroxylase thus blocking conversion of tryptophan to 5-hydroxytryptophan (Lovenberg *et al.*, 1967; Koe and Weissman, 1968). This is the rate limiting step in 5-hydroxytryptamine or serotonin biosynthesis. CIPhe also inhibits phenylalanine hydroxylase activity (Guroff, 1969); this may affect the synthetic pathway leading from phenylalanine through tyrosine and dopa (dihydroxyphenylalanine) to the catecholamines, i.e. dopamine, norepinephrine and epinephrine.

DEDTC inhibits conversion of dopamine to norepinephrine by inhibiting dopamine-β-hydroxylase (Goldstein *et al.*, 1964). Because noropinephrine is the immediate precursor of epineph-

rine, synthesis of the latter is also affected. DEDTC also inhibits monoamine oxidase (Nara *et al.*, 1966), and tyrosinases (Pomeranz, 1963).

The third monoamine antagonist, propranolol, binds to β-adrenergic receptors, thus interfering with the actions of norepinephrine and epinephrine. However, there is some evidence that it may bind to serotonin receptors as well (Sprouse and Aghajanian, 1986).

Besides acting specifically on the synthesis or function of monoamines the antagonists may also have undefined toxic effects when administered in high concentrations. Incubation of the newt blastulae in 1 mM of either CIPhe, DEDTC or propranolol prevented gastrulation and caused disintegration of the embryos. In more diluted solutions development proceeded but morphogenetic movements were either arrested at a later stage of gastrulation or morphogenesis led to anomalies of various degrees depending on the dose and duration of the treatment . During gastrula and neurula stages the embryos often expelled cells in different proportions. Severity of the consequent malformations in the larvae was related to the extent of cell loss.

Viable specimens of the experimental series were fixed on the 15th day of cultivation and divided into four groups (Fig. 1): Group A consisted of dwarfed, poorly-pigmented embryos lacking most organ rudiments. Group B consisted of anencephalic larvae lacking gills and limbs. Larvae in group C were microcephalic, eyeless, cyclopic or microphthalmic with poorly-developed gills and limbs. Larvae of these three groups lost a large number of cells in the early stages of development. Larvae in group D expelled a negligible number or no cells; this group was considered to exhibit the specific effects of respective monoamine antagonists and was chosen for histological and electron microscopical studies.

Below we summarize the results of experiments where the embryos were treated with monoamine antagonists for 48 hours from the late blastula through nerula stages.
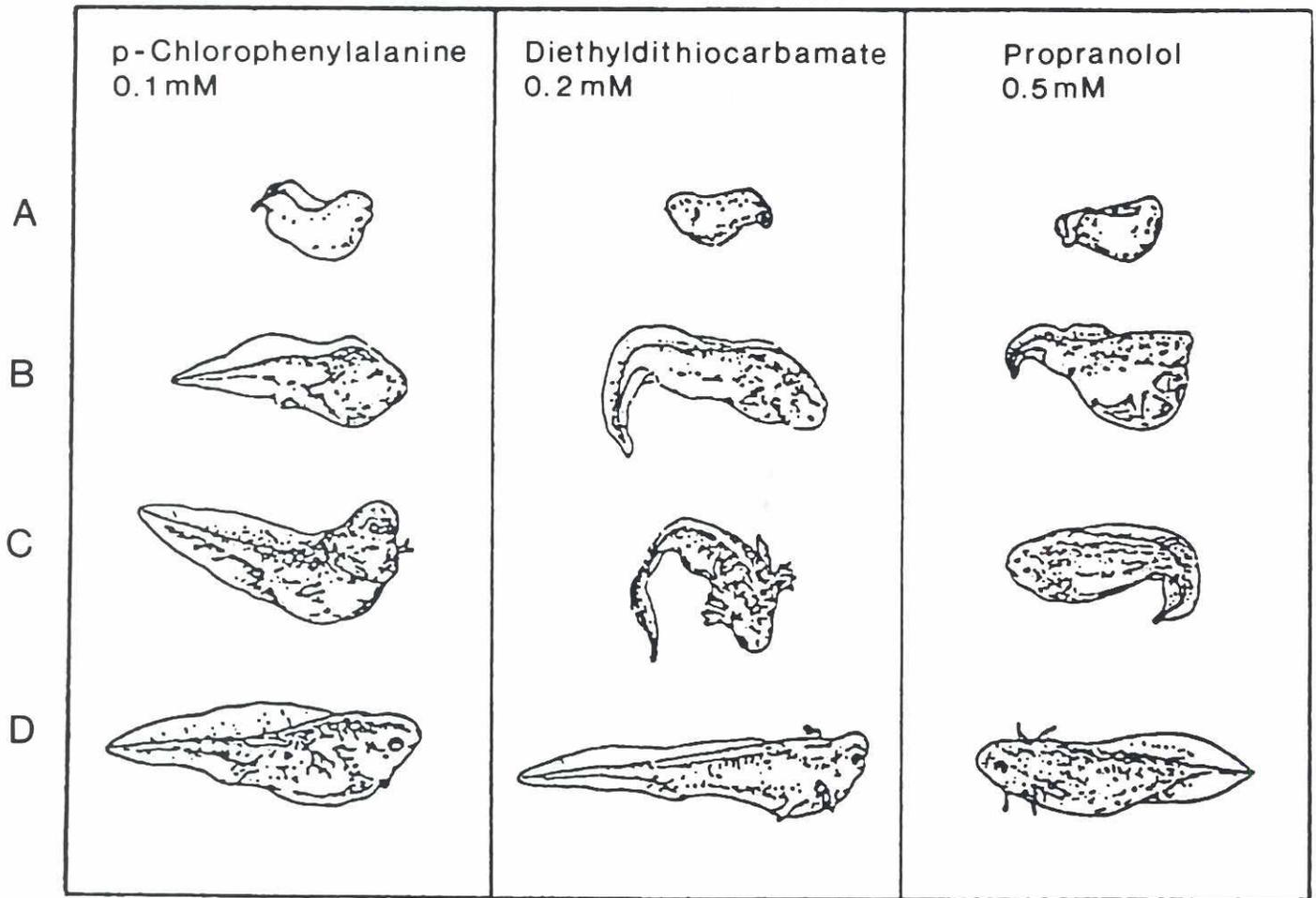
	p-Chlorophenylalanine 0.1mM	Diethyldithiocarbamate 0.2mM	Propranolol 0.5mM
A			
B			
C			
D			

Fig. 1. *Gradation of malformed 15-day old newt larvae treated with serotonin antagonists for 48 hours from late blastula through neurula stages.*

p-Chlorophenylalanine (ClPhe)

In 0.5 mM ClPhe gastrulation was arrested in its early phase; embryos lost cells and died within two days. Lower ClPhe concentrations retarded but did not prevent gastrulation. Embryos incubated for 48 hours in 0.1 mM ClPhe reached the yolk plug stage only slightly later than the controls. However, dissection of the gastrulae showed that involution of the prospective foregut endoderm and lateral expansion of the mesoderm were clearly retarded. At the late gastrula stage a thin-walled vesicle appeared on the anterio-ventral side of most embryos, probably because ectoderm without support of the underlying mesoderm yielded to the increasing fluid pressure in the diminishing blastocoele. At the neurula stage these vesicles usually disappeared or formed two lateral vesicles in the gill region. Although morphogenesis of some of the embryos in this experimental group was near normal, all of them developed more slowly than the controls, e.g. development of pigmentation was delayed for one to two days (Fig. 2).

The larvae grew stunted, microcephalic and often microphthalmic (Fig. 3). Some of them had two pulsating hearts.

Histological observations showed that ClPhe affects the development of the notochord: in experimental animals its diameter was smaller and the notochordal sheath thinner than in the controls. Utilization of the yolk was also retarded: yolk granules filled the notochordal cells of 15 day old treated larvae, but had disappeared from the controls (Fig. 4b). Electron micrographs revealed that collagen fibrils were sparse and disoriented in the notochordal sheath, and its outer elastic layer had developed poorly (Fig. 5b).

The anatomical anomalies could be traced back to the retarded cell movements rather than to impaired capability of the cells to differentiate or induce differentiation in other cells. For instance, ear vesicles were always present despite defects in the brain. The reason for this may be that ClPhe treatment affects morphogenetic movements necessary for the formation of the brain vesicles and optic cups, but does not interfere with the inductive actions of the neural tube. Two observations support this hypothesis: when gastrula ectoderm and blastoporal lip were combined, differentiation was identical in both ClPhe-treated and control explants; on the other hand, the curling up process of isolated ectoderm, which simulates the epiboly of ectoderm in gastrulation, was significantly retarded in the ClPhe-treated explants (Fig. 6).

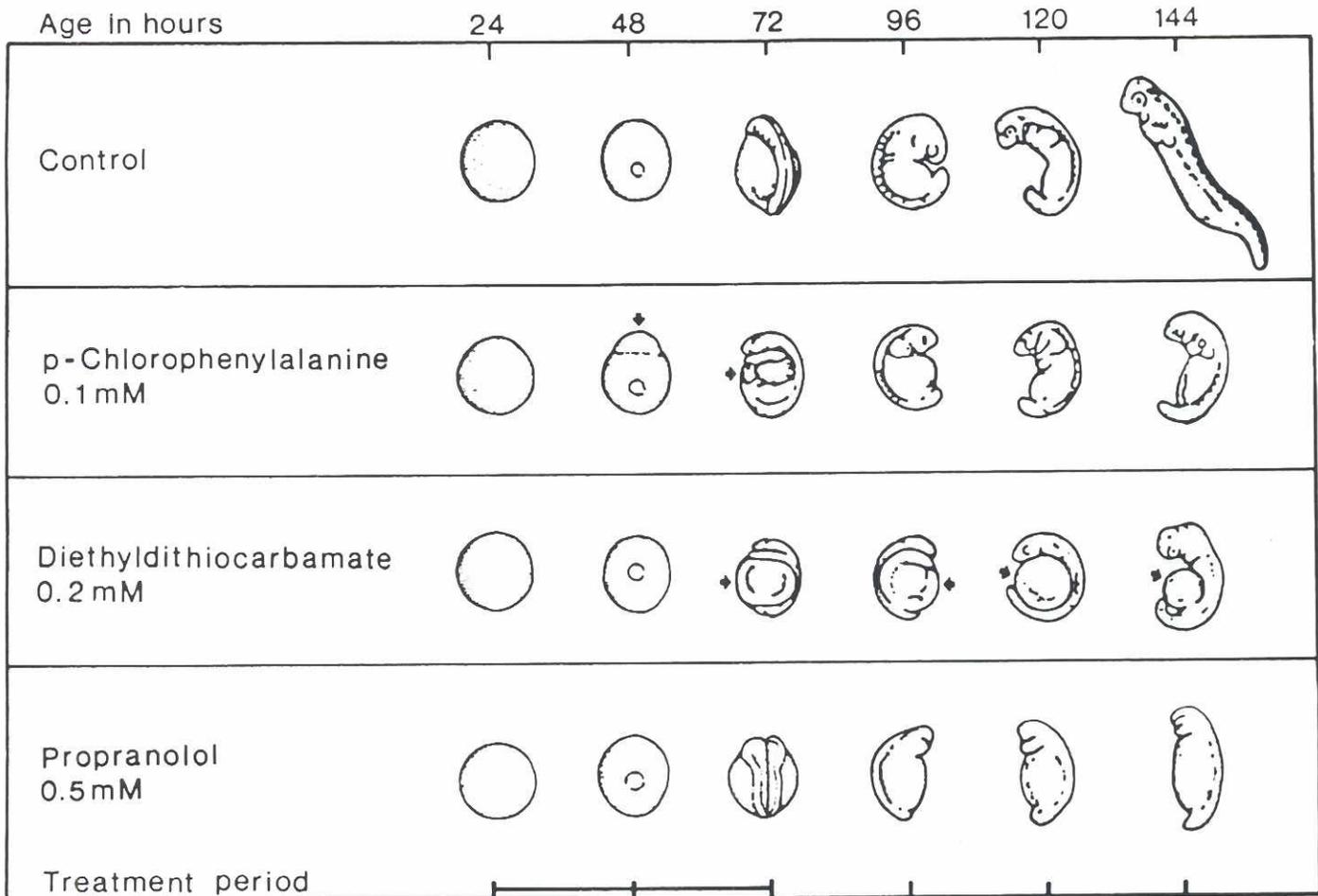The double hearts observed in several larvae might likewise

Fig. 2. *The rate of morphogenesis of control embryos and embryos treated with monoamine antagonists. Arrows point to the vesicles described in the text.*

be caused by delayed spreading of the anterio-ventral edges of the mesodermal mantle and consequent incomplete fusion of the differentiating heart primordia.

The mechanism underlying the effect of ClPhe treatment on the morphogenetic movements in the newt embryo is not known. In the sea urchin embryo, the first phase of gastrulation is inhibited by ClPhe treatment (Gustafson and Toneby, 1970, 1971). Because serotonin initiates the invagination of the ventral epithelium of the sea urchin blastula (Gustafson , 1973), it is probable that the main target of ClPhe is the serotonin biosynthesis.

It has been shown that serotonin might also have a role in vertebrate morphogenesis: it stimulates cell movements in explants from frog neurulae and tailbud embryos (Martynova and Belousov, 1978) and regulates expansion of the chick blastoderm (Palén et al., 1979).

On the other hand, serotonin treatment interferes with the morphogenetic movements of the chick embryo and in some cases prevents fusion of the heart primordia resulting in formation of two hearts (Palén et al., 1979). Results from experiments with chick and newt embryos are not necessarily contradictory: according to the hypothesis of Eiduson (1966), serotonin regu-

lates its own concentration by a feedback mechanism. Thus administration of extracellular serotonin may inhibit its intracellular synthesis.

In addition, serotonin treatment of the chick embryo noticeably hinders intracellular yolk degradation (Palén et al., 1979). Since it has been shown that serotonin is synthesised in the yolk granules (Emanuelson, 1974, 1976), it is plausible that extracellular serotonin inhibits transformation of yolk tryptophan to intracellular serotonin, which in turn may affect utilization of other components in yolk granules. This hypothesis may explain why ClPhe inhibits yolk granule degradation in the notochordal cells of the newt embryo.

Diethyldithiocarbamate (DEDTC)

Embryos incubated in 0.2 mM DEDCT completed gastrulation and their morphogenetic movements proceeded, although more slowly than in the controls (Fig. 2). The forward migration of the involuted endodermal cells was especially retarded; as a consequence fluid-filled ventral vesicles appeared at the neurula stage in several embryos; later the vesicles either diminished in size or burst expelling some cells. This cell loss did not have a significant effect on further morphogenesis. Larvae in group

D (Fig. 1) were often microcephalic with asymmetrical anterior structures: even an eye, a limb or gills could be missing from one side of the animal.

As compared with results in the CIPhe-treated group, DEDTC treatment led to slimmer and longer larvae, although they were slightly shorter than the controls. Pigment formation was delayed between one and two days.

The most prominent and least variable feature was hypertrophy of the notochord (Fig. 4c). Utilization of yolk in the notochord was normal --most of the yolk granules had disappeared by the 15th day of development.

Electron microscopical examination did not reveal any unusual structures in the DEDTC-treated embryos, except that the notochordal sheath was somewhat thinner than normal (Fig. 5c).

The mechanisms by which DEDTC interferes with amphibian morphogenesis are rather ambiguous. As a dopamine-β-hydroxylase inhibitor, DEDTC affects the catecholamine synthesis, but it may affect the serotonin metabolism as well: in sea urchin embryos gastrulation could be arrested with DEDTC, but its effect was prevented with simultaneous administration of 5-hydroxytryptophan, an immediate precursor of serotonin (Gustafson and Toneby, 1970).

Besides directly affecting catecholamine or serotin metabolism, DEDTC may also indirectly interfere with the synthesis and release of monoamines since they modify the synthesis and release of each other through feedback mechanisms.

It has been suggested that catecholamines might provide the motive force for morphogenetic movements in the chick embryo (Lawrence and Burden, 1973); thus both serotonin and catecholamines might regulate morphogenesis even in vertebrates as they do in echinodermates.

Hypertrophy of the notochord caused by DEDTC treatment cannot be explained by a simple decrease in the serotonin synthesis because CIPhe, which is a relatively specific inhibitor of tryptopham hydroxylase, retarded both the development of notochord and the utilization of its intracellular yolk. If the main effect of DEDTC on the synthetic pathway of monoamines is the inhibition of dopamine-β-hydroxylase activity, it would lead to a decrease in the norephinephrine concentration and an increase in dopamine and dopa concentrations. A change in the ratio of catecholamines and serotonin could be the factor triggering an increase in the chordamesodermal gradient. However, Palén *et al.* (1979) did not observe hypertrophic notochords in chick embryos incubated with DEDTC, which might indicate possible differences in avian and amphibian monoamine metabolism.

The delay in pigmentation is probably caused by inhibition of tyrosinase activity, but may also be caused by retardation of development including the migration melanophore progenitor cells from the neural crest.

Propranolol

Treatment of the embryos with 0.5 mM propranolol caused anomalies which were similar to those caused by CIPhe, i.e. retarded morphogenetic movements and stunted, microcephalic larvae (Figs. 1 and 2). Histological study did not reveal features which could be interpreted as a consequence specific for propranolol treatment. Propranolol did not affect the development
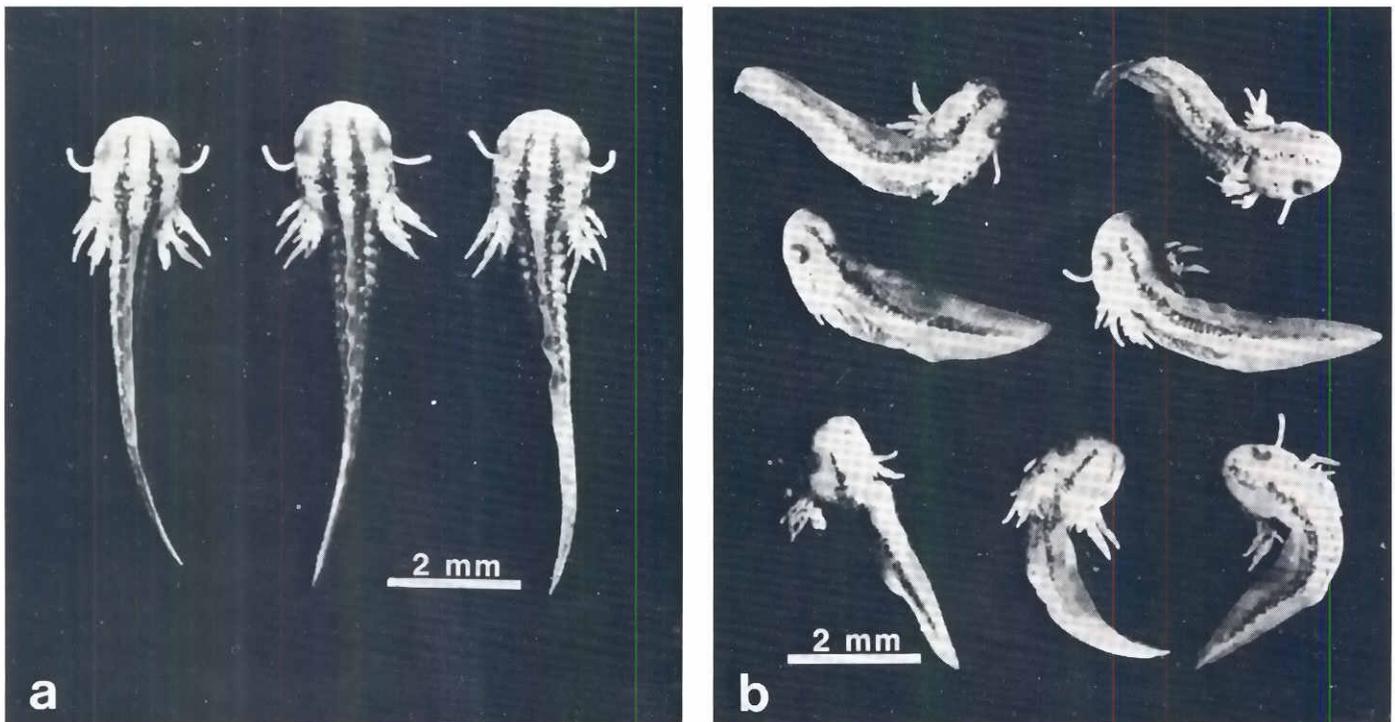

Fig. 3. *Newt larvae at the 15th day of development. (a) Controls, (b) larvae treated with 0.1 mM chlorophenylalanine for 48 hours beginning at late blastula stage.*
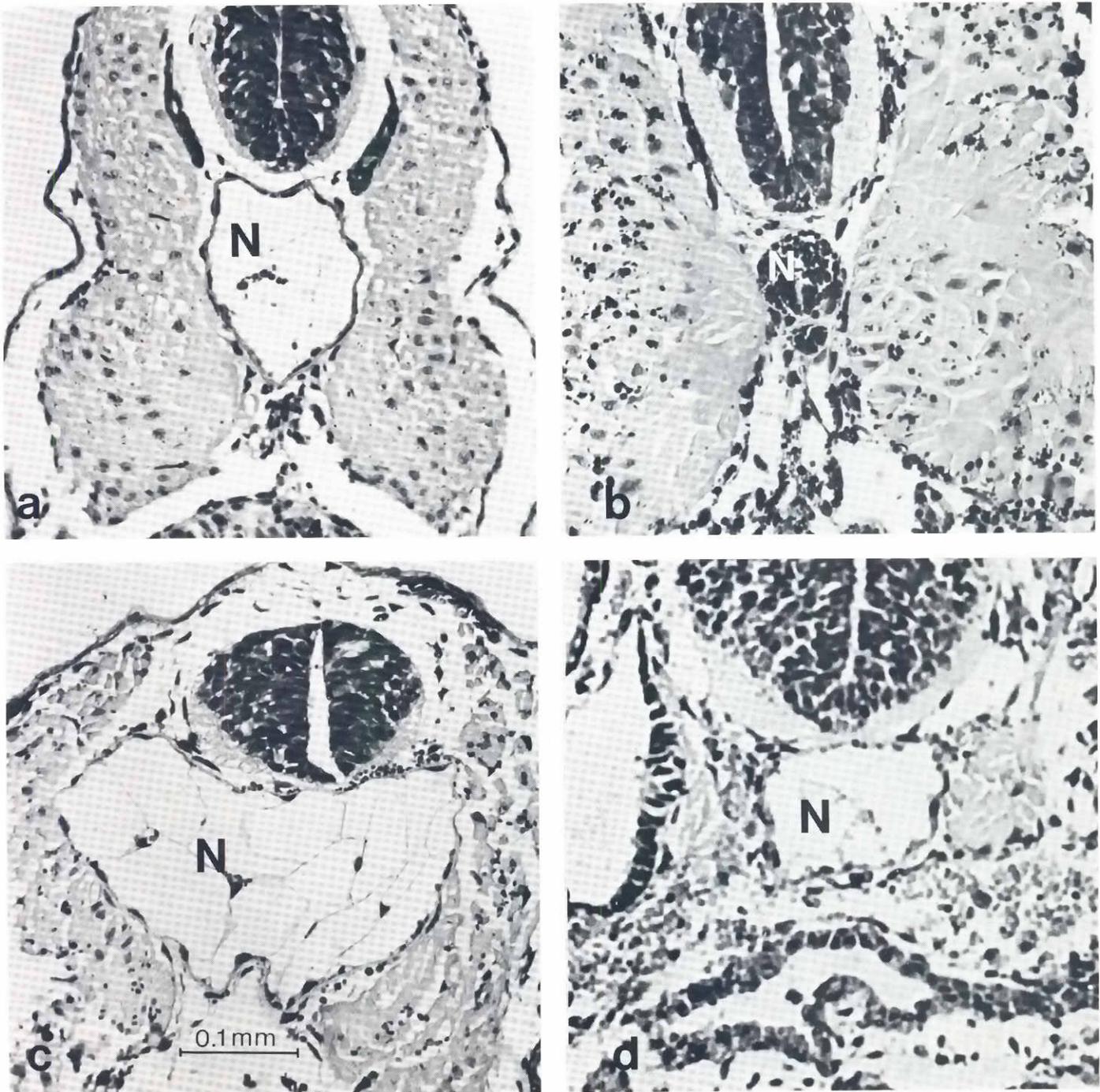
Fig. 4. *Photomicrographs of transverse sections of newt larvae reared for 15 days. (a) Control; (b) larvae treated with p-chlorophenylalanine; the notochordal cells are filled with yolk granules; (c) diethyldithiocarbamate-treated larva with hypertrophic notochord; (d) larva after propranolol treatment. All experimental larvae were treated from late blastula through neurula stages.*
N = notochord.

of notochord: neither its diameter nor the structure of the cells differed from the controls.

Exogenic adrenergic agonists —isoproterenol, norepinephrine and epinephrine— inhibit *in vitro* migration of epidermal cells of the adult newt (Donaldson and Mahan, 1984). The mechanism by which catecholamines exert their effect on epi-

dermal cell migration might be activation of adenyl cyclase and elevation of the level of cyclic adenosine monophosphate in the cell. Inhibition of the cell migration could be removed with the β-receptor-blocking propranolol.

These results seem to be in contradiction with the observations obtained with the newt embryos: delayed gastrulation and
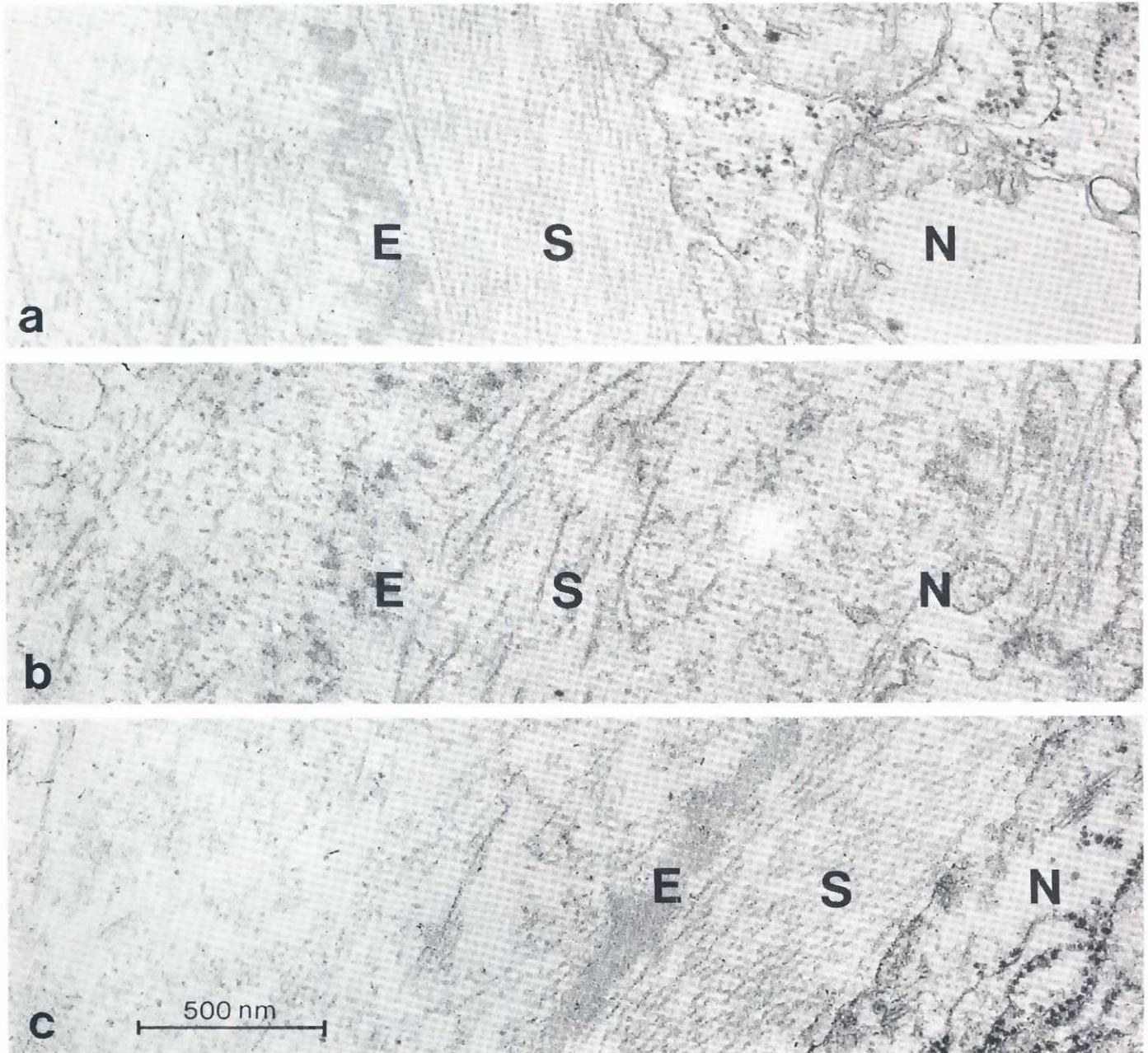
Fig. 5. *Electron micrographs showing notochordal sheath of newt larvae at the age of 15 days. (a) Control; (b) after p-chlorophenylalanine treatment; (c) after treatment with diethyldithiocarbamate.*
S = collagen sheath; E = elastic layer; N = notochordal cells.

stunted larvae suggest retarded rather than accelerated morpho-
genetic movements. However, a hypothesis proposed by
Donaldson and Mahan (1984) may explain the contradictory
results: cAMP either accelerates or restrains cell motility
depending on its concentration. Propranolol may repress the
morphogenetic movements by decreasing the level of cAMp in
the cells below a certain optimum. But for the specific response
to propranolol the cells must have specific binding sites;
whether they occur in the cells of early newt embryos is not
known.

Materials and methods

Embryos of the smooth newt, *Triturus vulgaris*, were treated with
either DL-p-chlorophenylalanine methylester hydrochloride (Calbio-
chem), Na-diethyldithiocarbamate (Merck) or DL-propranolol (Sigma).
Untreated embryos served as controls.

Experimental embryos were incubated for different periods of time
beginning at late blastula stage with the respective monoamine antago-
nist diluted in sterile, buffered Holftreter solution.

After treatment the embryos were reared in Holtfreter solution at
17 °C. Larvae were fixed at the age of 15 days and processed with rou-
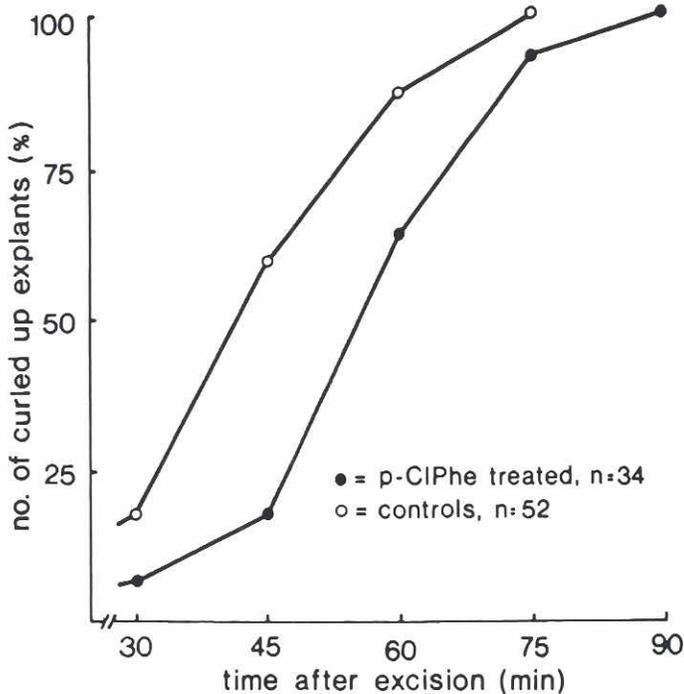tine methods for histological or electron microscopical studies.

Fig. 6. *Diagram showing the cumulative number (as percentage of total) of curled-up ectodermal explants at 15 min intervals.*

In the experiments with explants, pieces of ectoderm and blastoporal lip were excised from early gastrulae; the explants were then treated with p-chlorophenylalanine in the same way as the whole embryos.

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