The Effect of Benzimidazole on the Differentiation of Ectodermal Explants from the Gastrulae of *Xenopus laevis*

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WITH ONE PLATE

INTRODUCTION

THE treatment of amphibian and chick embryos during the early stages of development with small concentrations of benzimidazole, and some of its derivatives, results in the formation of abnormal embryos (Liedke, Engleman, & Graff, 1954; Waddington, Feldman, & Perry, 1955*a*; Billett & Perry, 1957 *a*, *b*). The gross effects produced by these substances are of a fairly general kind. Abnormal gastrulation, decomposition of neurulae, and microcephaly are produced in Amphibia. Rather less well-defined abnormalities, involving the head, neural tube, and somites, are seen in the chick.

The way in which these abnormalities are produced is not known. The idea that benzimidazole acts simply as a purine anti-metabolite is not well founded (Slonimski, 1954). The work of Tamm and his colleagues (Tamm, Folkers, Shunk, & Horsfall, 1953; Tamm, 1958) has shown that benzimidazole and certain of its derivatives inhibit the growth of some viruses. The nature of some of these derivatives, and some recent biochemical observations (Allfrey *et al.*, 1957; Tamm, 1957), suggest strongly that the compounds interfere in some way with ribonucleic acid metabolism. Although there is no direct evidence to confirm the view, it is conceivable that the abnormalities produced in embryos by benzimidazole and its derivatives are caused primarily by disturbances in ribonucleic acid metabolism.

Examination of newt embryos treated with benzimidazole at the neurula stage shows that cell degeneration is particularly evident in the neural tissue and is also seen to some extent in the newly formed mesenchyme (Waddington *et al.*, 1955*a*). A similar pattern of damage was seen in *Xenopus* and chick embryos treated with benzimidazole and its alkyl derivatives (Billett & Perry, 1957 *a*, *b*).

Although the localized cell damage seen in the intact embryos may be the

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result of the direct effect of benzimidazole on the cells, this is by no means certain. The compound may act by reducing the metabolic activity of all the cells in the embryo, and disintegration might then start in localized areas of cells most sensitive to a general toxic effect. Dividing or differentiating cells might come within this category. Alternatively, abnormalities of gastrulation might occur and neurulation could then be upset by the disturbance of normal inductive relationships. For instance, an embryo having undergone a partial exogastrulation might develop a retarded head and show extensive cell damage in the forebrain region.

Difficulties of interpretation can be overcome to a certain extent by using isolated pieces of embryonic ectoderm capable of a limited amount of differentiation. Such explants, readily obtained from amphibian gastrulae, are relatively simple systems in which a small number of well-defined cell types arise during the limited life of the explants. Compared with whole embryos the explants are small and their external surface is uniform. Under these conditions a fairly rapid and even penetration of benzimidazole is to be expected.

The following account describes the effect of benzimidazole on the differentiation of ectodermal explants prepared from the late gastrulae of *Xenopus laevis*. These effects are compared with the action of benzimidazole on explants not capable of differentiation, prepared from early gastrulae.

EXPERIMENTAL

Fertilized eggs were obtained from the toads by injection of chorionic gonadotropin. Capsules and vitelline membranes were removed, and the embryos transferred to full-strength Holtfreter's solution, where the explants were made. Sterile conditions were maintained during the operations and for the subsequent culture of the explants.

The explants from early gastrulae (stage 10–10½ of Nieuwkoop & Faber, 1956) consisted of the ectoderm covering the blastocoel cavity and excluded the dorsal lip and any invaginated material. The explants from late gastrulae (stage 11½–12 of Nieuwkoop & Faber) were prepared similarly and consisted of the ectoderm covering the remains of the blastocoel cavity and that in contact with the archenteron roof. Care was taken to remove adhering cells of the underlying tissue.

As soon as the explants were made they were placed either in full-strength Holtfreter's solution or in Holtfreter's solution containing 100 μg./ml. of benzimidazole. About half the treated explants were placed in full-strength Holtfreter's solution before transfer to the solution containing the benzimidazole (delayed treatment). This was to allow the formation of a closed external surface. The solutions were buffered either with phosphate or with bicarbonate to a pH of 6·8–7·2. The explants were cultured on a layer of 2 per cent. agar. Under these conditions the concentration of benzimidazole in the test solutions was found to fall, owing to its absorption by the agar. The solutions were renewed

about every 24 hours, giving an effective concentration of benzimidazole between 50 and 100 μg./ml.

The explants were removed and fixed in Bouin's mixture after approximately 48, 60, and 72 hours. Serial sections of 5–7 μ were cut. The sections were stained either with pyronine and methyl green or with haematoxylin.

RESULTS

The number of explants made, their types, the number which survived, and the extent of their differentiation are given in Table 1.

TABLE 1

Differentiation of ectodermal explants from gastrulae of Xenopus laevis

Type	*Duration of culture (hours)*	*No. made*	*No. survived*	*Differentiation*			
				Ectoderm only	*Neuroid*	*Neural*	*Mesenchyme*
Early gastrulae	68	Cont. 26	6	6	0	0	0
		Test 30	7	6	0	0	0
Mid- to late gastrulae	48	Cont. 30	19	9	5	4	0
		Test 32	24	9	5	10	0
		Test (D) 30	25	13	8	4	0
Mid- to late gastrulae	60	Cont. 31	24	1	11	12	5
		Test 33	20	3	10	8	2
Mid- to late gastrulae	72	Cont. 22	22	8	1	12	8
		Test 24	23	7	4	11	9
		Test (D) 15	13	4	1	8	4

Cont. = Holtfreter's solution.

Test = Holtfreter's solution+benzimidazole 50–100 μg./ml.; explants placed in solution immediately.

Test (D) = Holtfreter's solution+benzimidazole 50–100 μg./ml.; explants placed in solution 3 hours after preparation, to allow healing.

Explants were considered to have undergone neuroid differentiation if they possessed an internal mass of cells which was distinct from the surrounding ectoderm (Plate, fig. A). This cell mass was characterized by the following features. It possessed a vague structure, the cells being roughly orientated about the centre of the mass. The cells of the mass were smaller and more heavily stained than those of the epidermis. Mitoses were observed frequently in the neuroid mass; in the epidermis mitoses were rare. Neural differentiation, which is invariably of the archencephalic type in these explants, was considered to be present when distinct structures composed of neuroid cells were seen (Plate, fig. E).

The explants made from the early gastrulae did not undergo differentiation. In these explants the survival was poor, both in the tests and in the controls. After 68 hours' culture no differences could be detected between the treated and untreated explants, either in terms of survival or in histological appearance.

In addition to the explants from the early gastrulae, about a third of the explants from the middle to late gastrulae failed to differentiate. In these cases, too, no difference could be detected between the test and control explants.

Benzimidazole neither prevented neural differentiation nor did it noticeably affect the survival of explants prepared from middle to late gastrulae. This is clearly shown by the facts which are summarized in Table 2. Although benzimidazole did not prevent differentiation, the neural tissue which was formed

TABLE 2

Effect of benzimidazole on the survival and differentiation of Xenopus *ectodermal explants from mid to late gastrulae*

(Cultured 48–72 hours; figures are percentages)

	Benzimidazole 50–100 μg./ml.	*Controls*
Survived	78	79
Neuroid differentiation	28	26
Neural differentiation	39	43

TABLE 3

Location of damaged cells

Xenopus ectodermal explants mid- to late gastrulae. Benzimidazole 50–100 μg./ml.

	48		60		72	
Duration of culture (hours):	*Cont.*	*Test*	*Cont.*	*Test*	*Cont.*	*Test*
No. of explants showing neuroid–neural differentiation:	9	27	23	18	13	24
Cell damage: Between neuroid mass and ectoderm	0	5	13	14	0	10
Cell damage: Closely investing neuroid mass	0	1	0	7	0	6
Cell damage: Scattered in neuroid mass	0	6	1	15	0	21

was clearly affected by the treatment. The location of the damaged cells in the explants which had undergone neuroid or neural differentiation is given in Table 3. Cells which were presumably dead or dying were revealed by abnormally intense staining. Such cells were localized mainly in three places. They were seen between the differentiating neural mass and the ectoderm, closely investing the neuroid mass, and scattered throughout the neuroid or neural structure itself. Abnormally stained cells were also seen in the control explants, but they were not very numerous and tended to be scattered throughout the sections. After about 48 hours' culture about a third of the treated explants contained damaged cells. At 60 hours nearly all the treated explants were

affected. At 72 hours, in addition to the neural tissue, the ectomesenchyme was affected.

The most striking pattern of cell damage seen in some of the treated explants was a ring of material heavily stained with pyronine and closely investing the differentiating neural mass. This material appeared to consist of two components. One was a layer of shrunken cells between the neural structure and the ectoderm. In the living material this component was probably a layer of dead or dying cells surrounding the spherical mass of the differentiating neural cells, lying beneath the adhesive gland. The second component consisted of the distal portion of the cytoplasm of the cells on the outside of the neural structure.

In corresponding areas of control sections the border between the ectoderm and the differentiating neural mass was sometimes seen to be demarcated by a thin line of pyronophilic material, consisting of the borders of the cells on the periphery of the neural mass. Compared with the treated sections, however, this area was much less intensely stained. The contrast in the appearance of these areas in test and control sections is seen in figs. A, B, C, D of the Plate.

After 48 and 60 hours' culture, sections of many of the treated explants revealed pycnotic and heavily stained cells in the developing neural tissue. After 72 hours the neural tissue of the treated explant showed extensive damage (Plate, figs. E, F). In addition to cells containing pycnotic nuclei, these contained many cells in which the nuclei were abnormally large and lightly stained. These large nuclei are shown in fig. G of the plate, where they may be compared with nuclei in a corresponding area of a control explant (Plate, fig. H).

The cytological appearance of the epidermis appeared to be identical in the treated and untreated explants. After 60–70 hours the ectoderm taken from middle to late gastrulae became ciliated. At 72 hours the ciliated epidermis was sufficiently developed to propel some of the explants over the agar base in the culture dishes. Movement of this kind was shown by both the treated and untreated explants.

DISCUSSION

The results indicate that the neural tissue and the ectomesenchyme which form in the explants are selectively damaged by benzimidazole, the effect being very similar to that seen in whole embryos. The epidermis does not appear to be affected by the treatment. Explants which have been in contact with benzimidazole for as long as 3 days possess epidermis which cannot be distinguished either histologically or functionally from that of the controls.

It is especially interesting to note that the effect of benzimidazole is only revealed as cell damage after about 2 days, when about half the explants prepared from middle to late gastrulae have begun to differentiate. Only about a quarter of these explants show signs of cell damage. This delayed effect may be due either to a slow build-up of a toxic concentration of benzimidazole inside the explant, or to the absence of susceptible cells when the explants were made.

A delayed effect seems to be ruled out from observations on intact embryos, where toxic effects are observed after about 24 hours. Bearing in mind that explants are very much smaller than entire embryos, it seems reasonable to assume that the cells of the neuroid mass in the explants will develop in a concentration of benzimidazole which would have proved lethal to embryos.

When differentiation starts in the explants neither the formation of the neuroid mass nor its further development into a neural structure of some kind is prevented by benzimidazole. However, cytological damage is observed soon after differentiation has begun and becomes widespread as differentiation proceeds. A marked feature of the differentiation areas is the presence of numerous mitoses. Cell division proceeds in the presence of benzimidazole. A count of the number of mitoses in the test and control explants at 60 hours indicated that, at this stage, mitosis was unimpaired in the treated explants. At 72 hours, on the other hand, there were fewer mitoses in the treated explants than there were in the controls. This is almost certainly a secondary effect.

In some of the treated explants abnormally high concentrations of pyronophilic material were seen in the cytoplasm of the neuroid cells on the periphery of the neuroid mass. This type of cytoplasmic staining does not appear to be related specifically to the action of benzimidazole. Waddington (1958) has described the formation of strongly pyronophilic lumps of material in the cytoplasm of the neural cells of amphibian embryos treated with tri-ethyl melamine and myleran, and he suggests that such globules may indicate an overproduction of ribonucleic acid.

At 60 hours the most characteristic abnormality seen in the treated explants was simply shrunken, pyronophilic, cells. At 72 hours, however, in addition to pycnotic cells, many of the cells of the neural tissue appeared enlarged and contained large nuclei which were poorly stained. It must be emphasized that this latter cytological appearance occurs after the pycnotic abnormalities have become well established. Thus any suppression of cell division and consequent enlargement of interphase nuclei must be regarded, in this case at least, as secondary and possibly the result of toxic effusions from neighbouring moribund cells. These cytological appearances reveal only that some of the cells were killed, either directly or indirectly, by benzimidazole. We learn nothing about the original biochemical lesion.

More significant than the pathological appearance of individual cells is the overall pattern of cell damage. In the treated explants the highest concentration of damaged cells undoubtedly surrounds the neural mass. This is indicated in the earlier stages by the appearance of a pyronophilic ring surrounding the neural mass and later by a mass of degenerating cells lying between the epidermis and a poorly organized neural structure. A pattern of cell damage of this kind is consistent with the idea that cells in the process of differentiation, following an initial phase of mitoses, are especially sensitive to the action of benzimidazole.

The biological effects of benzimidazole and its derivatives need to be

interpreted with extreme caution. The compounds have diverse effects on a number of apparently very different systems. Some derivatives produce general toxic effects. For instance, the dichloro-ribazole derivative reduces oxygen uptake and causes cell damage in the chorio-allantoic membrane of the chick (Tamm, 1956). Administered to mammals, benzimidazole acts as a muscle depressant (Goodman, Gillman, & Hart, 1943; Goodman & Hart, 1944). The swimming activity of amphibian larvae is quickly inhibited by benzimidazole and its alkyl derivatives (Billett, 1958). Further, there is some evidence to suggest that benzimidazoles are metabolic antagonists of vitamin B_{12} (Arscott, Shorb, & Boggs, 1955).

However, the concentrations of benzimidazole and its derivatives required to produce these general toxic and pharmacological effects are relatively high, and there is good reason to believe that the benzimidazoles can act selectively if used at sufficiently low concentrations. Tamm and his colleagues have shown convincingly that benzimidazole can reduce the yield of influenza virus grown on chick allantois without noticeable effects on the uninfected host cells. In the case of *Xenopus* explants, culture in benzimidazole results in gross cytological damage in the central differentiating mass. A high concentration and rapid turnover of ribonucleic acid appears to be a feature of differentiating cells, and very similar conditions probably exist in cells supporting the growth of RNA-containing viruses. In both cases the deleterious action of benzimidazole and its derivatives could be due to one and the same cause, a selective disruption of RNA metabolism.

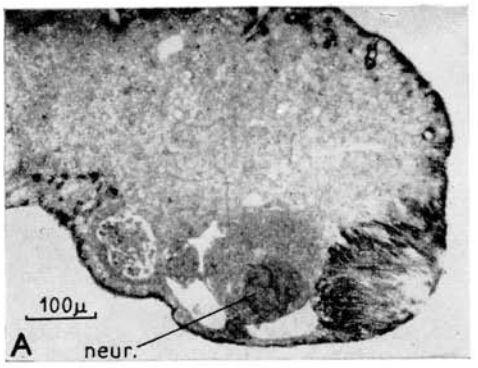
SUMMARY

The survival of ectodermal explants taken from early to late gastrulae of *Xenopus laevis* does not appear to be affected by culturing them, for up to 3 days, in solutions containing 50–100 μg./ml. of benzimidazole. This compound does not prevent neural differentiation. Cell damage only becomes apparent after about 48 hours when it is confined to the differentiating neural tissues and the surrounding cells. The histological appearance of the ectoderm cells and the fact that they develop functional cilia suggest that they are not affected by the treatment. The results suggest that tissue in the process of differentiation is highly susceptible to benzimidazole. This susceptibility may be a consequence of the high rate of turnover of RNA in differentiating tissue.

RÉSUMÉ

Effet du benzimidazole sur la différenciation de fragments d'ectoderme prélevés sur des gastrulas de Xenopus laevis

La survie des fragments d'ectoderme prélevés chez *Xenopus laevis* depuis le début jusqu'à la fin de la gastrulation ne paraît pas affectée par leur culture, dans les limites de trois jours, dans des solutions contenant 50 à 100 μgr. de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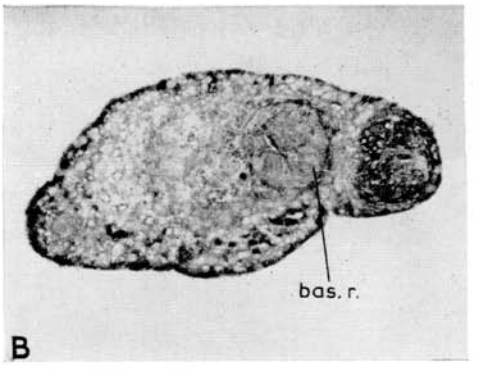
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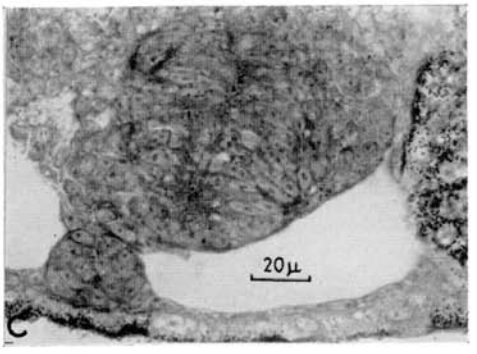
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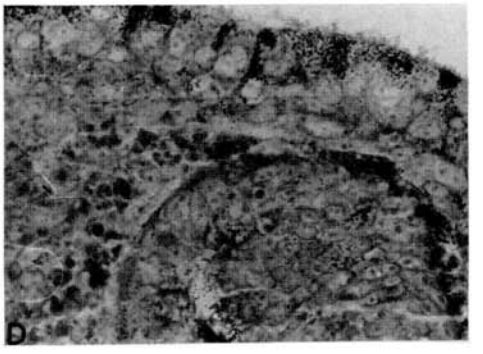
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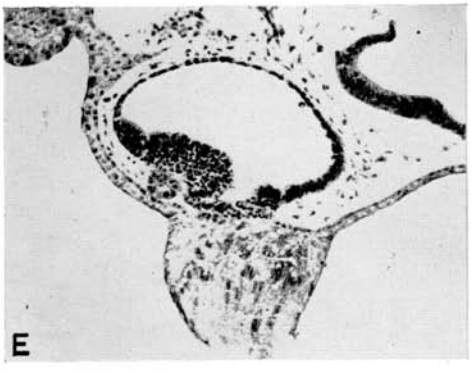
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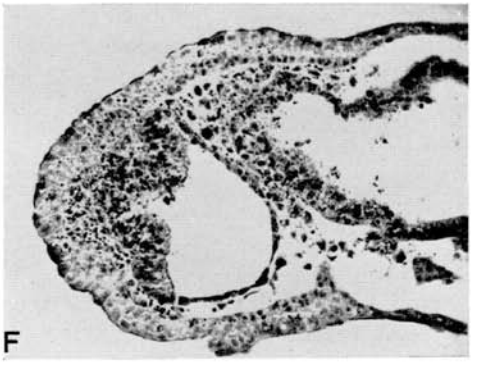
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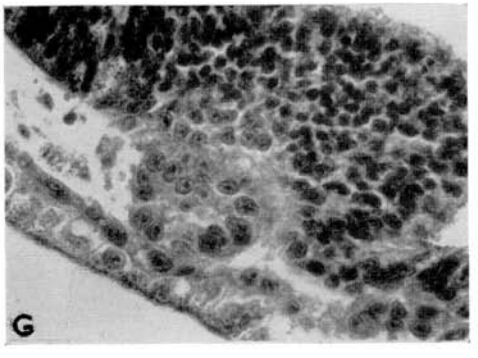
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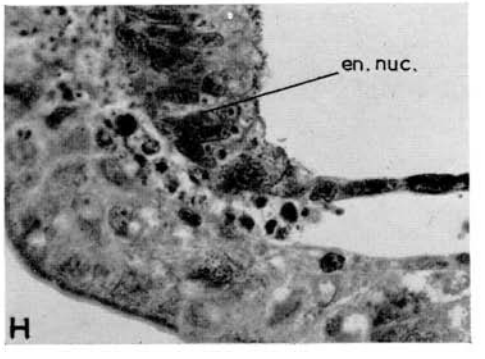
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benzimidazole par ml. Ce composé n'empêche pas la différenciation neurale. Des lésions cellulaires ne deviennent apparentes qu'après environ 48 heures et sont alors limitées aux tissus neuraux en différenciation et aux cellules voisines de ceux-ci. L'aspect histologique des cellules de l'ectoderme, et le fait qu'elles se garnissent de cils fonctionnels suggèrent qu'elles ne sont pas affectées par le traitement. Les résultats suggèrent qu'un tissu en voie de différenciation est très sensible au benzimidazole. Cette sensibilité peut être une conséquence du métabolisme intense de l'ARN dans le tissu en différenciation.

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EXPLANATION OF PLATE

FIG. A. Section of explant cultured for 60 hours in Holtfreter's solution. Note neuroid differentiation (*neur.*).

FIG. B. Section of explant cultured for 60 hours in Holtfreter's solution containing 50–100 μg./ml. benzimidazole. Note the basophilic ring (*b.r.*) surrounding the differentiating cell mass.

FIG. C. The section shown in fig. A at a higher magnification, showing the neuroid mass of cells.

FIG. D. The section shown in fig. B at a higher magnification, showing part of the basophilic ring.

FIG. E. Section of explant cultured for 72 hours in Holtfreter's solution, showing neural differentiation.

FIG. F. Section of explant cultured for 72 hours in Holtfreter's solution containing 50–100 μg./ml. benzimidazole, showing poor development of neural structure.

FIG. G. Section shown in fig. E at a higher magnification.

FIG. H. Section shown in fig. F at a higher magnification. Note enlarged nuclei in neural tissue (*en. nuc.*).

All sections were cut at 5–7 μ. Specimens A, B, C, and D stained with methyl green and pyronine; specimens E, F, G, and H stained with haematoxylin.

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