CHAPTER 8

Embryonic Growth in Animals

8.1: Action on Gonads and Early Development

Eggs have often proved to be an excellent material for colchicine research, and in previous chapters results of work on various types of eggs have been mentioned. Nuclear structure is modified in *Tubifex*,[22, 23] the nuclear sap becomes granular in the *Anodonta* egg,[11] spindle changes are most evident in *Arbacia*,[4, 41] disturbances of cleavage are noted in *Sphaerechinus*,[6] while curious surface changes have been described in both *Tubifex*[44] and *Arbacia*.[26] The size of egg cells, their conspicuous spindle, and the possible induction of polyploidy were factors making them useful in some of the early colchicine research. It is remarkable, however, that the first paper on this subject was written by two botanists.[27]

We shall consider here only facts which have not been observed in ordinary cells, and which are related to the special physiology and cytology of eggs. Since there are few papers on modifications of spermatogenesis, it was thought natural to describe some of the results which may prove important for the possible induction of polyploidy in animals. This last problem will be discussed more thoroughly in Chapter 16. On the other hand, the disturbances of embryonic growth related to mitotic poisoning result in some quite peculiar malformations which will be considered later in this chapter.

8.1–1: The cleavage of eggs. All work in this field points towards the complexity of colchicine actions, which are not only related to the stage of maturation or growth reached by the eggs or the young embryo, but also to the concentrations of alkaloid used. For instance, in some of the early work on the egg of *Rana pipiens* the classification of cellular changes proved to be very difficult because of great differences of sensitivity.[18] A 1:1000 solution suppressed all cleavage and led to cellular disintegration; at 1:10,000, colchicine did not disturb the first cleavage, but the next ones were irregular and the grooves between the cells were only shallow; at 1:100,000, three cleavages proceed normally, but in many eggs the grooves faded away later. Even when the concentration was only 1:1,000,000 and when some apparently normal embryos grew, abnormal cleavages were visible, and on the third day all the embryos were found dead. It was evident that even when nuclear mitosis proceeded normally, cleavage could be inhibited. Gastrulation was made impossible, the eggs assuming a meroblastic type of growth.

It was soon discovered that in *Arbacia* the sensitivity of the eggs decreased rapidly after fecundation;[4] 40 minutes later, from 90 to 100 per cent of normal cleavages could be observed. In the sea urchin *Paracentrotus,* before fecundation, the eggs may live only in a 1:200,000 solution. Later, cleavage is quite abnormal. If colchicine is applied at fecundation, a 1:60,000 solution does no more than disturb gastrulation. A temperature effect was also observed. Inhibition of growth was nearly complete if colchicine had been allowed to act at 25°C., even if the eggs were kept at lower temperatures later. On the contrary, colchicine at 15°C. permitted growth to the morula stage, or, if the eggs were placed at 25°C. after colchicine, as far as the 16-celled stage. This temperature effect was tentatively related to permeability changes.[30]

The peculiar behavior of egg cells and the first stages of development of amphibia have been the subject of a thorough analysis, related in many papers of the French author, Sentein.[34, 35] Like other workers, he found that cleavage disturbances were not closely related to mitotic disturbances; precocious cleavage could, in some eggs, lead to anucleate blastomeres. The complexities of the action of colchicine are revealed by the various cytological anomalies described: polyploidy, plurinucleation, asymmetrical development, chromatin bridges between nuclei, pycnosis, and pluricentric mitoses. The last were found during recovery and are comparable to the multiple stars described in Chapter 3.

The variable reactions during development were analyzed in *Triturus, Pleurodeles, Bufo, Rana,* and *Amblystoma.*[34] After gastrulation, typical arrested mitoses of the star type are the rule, with clumped chromosomes that are progressively destroyed. In the earlier stages, however, nuclear changes are quite different. Rather concentrated, 1:500 and 1:1000, solutions of colchicine were used. However, the cytological changes were always delayed, as observed by the other authors mentioned above.[4, 18, 41] First of all, cleavage is inhibited, the nucleus completing its division. The result of this is the frequent observation of binucleate blastomeres. The spindle may be completely destroyed; large, probably polyploid nuclei are found later. However, the normal number of chromosomes is most often maintained because the spindle, even in these high concentrations of colchicine, recovers. This leads often to pluripolar spindles, which are considered to be an important factor counteracting the polyploidizing action of the alkaloid. Recovery is incomplete, and chromosome counts demonstrated a great variability from cell to cell.[16]

Another peculiarity of the spindle of amphibian eggs is its asymmetrical reactions towards the depolarization effects of colchicine. The hypothesis has been put forward that this may be related to a differential sensitivity of the centrosomes, whether of paternal or maternal origin.[34]

Similar disturbances of development have been described in *Rana agilis*[9] and *Bufo vulgaris,* where an apparent decrease of cellular respiration was observed.[37] The exact relation between mitotic changes and the abnormalities of later development, which will be related in the next section, are most difficult to understand. A detailed description of the action of colchicine on the cleavage and early development stages of the fish *Oryzias latipes* cannot possibly be summarized here, but should be consulted by embryologists interested in chemically induced abnormal growth.[39]

The changes described in the egg of *Tubifex,* an invertebrate, are remarkably similar to those reported in vertebrates. In 1:30,000 solutions of colchicine some eggs are able to divide twice. One of the main effects is on cytoplasmic limits, which may disappear after having been normally formed at telophase.[44]

A relative resistance towards colchicine, changes in sensitivity related to developmental stages, the absence of polyploidy in the embryos, and peculiar actions on cleavage are the main facts which at this time emerge from a great amount of observations.[29, 40] There is no doubt that cytologists and embryologists have many more problems to solve and probably new types of colchicine effects to discover.

8.1–2: Male gametes. There are surprisingly few data available on the action of colchicine on spermatogenesis. In mice, aged 22 days, some arrested mitoses (or meioses?) have been reported in early work.[24] In adult animals, colchicine brought evidence of nuclear and cytological destruction. Arrested mitoses of spermatogonia in rats injected with more than 1.4 mg/kg of the drug have been described. The spermatocytes did not appear to be altered, although 24 hours after the injection the number of metaphases was somewhat increased.[32]

Personal observations of the junior author (unpublished) are that in the testes of mice injected 1.25 mg/kg, most of the spermatocytes have no more spindle 24 hours later. Spermatogonia appear to be unaltered, and the stages of meiosis are normal, as long as no spindle activity is required. Many spermatids with vacuolated nuclei may be observed, but this phenomenon is a consequence of the general toxicity of colchicine, and has been described under various experimental conditions and with other mitotic poisons.[38] With less toxic colchicine derivatives, spindle inactivation is apparent in a few hours. Depending on the doses injected, recovery is possible, or considerable cellular damage may be found. Binucleated spermatids may result from the spermatogonial mitoses during recovery assuming the "distributed" type with two nearly equal groups of chromosomes (cf. Chapter 2).

In fowls also, colchicine may induce severe degenerative changes in testicular cells. These are followed by regeneration seven days later.[17]

No polyploid spermatozoa have been reported in vertebrates. On the contrary, in the insect *Triatoma infestans* (order: Hemiptera), colchicine not only inhibits the spindle function, but as a consequence, modifies considerably the size of the spermatids (Fig. 8.1). This is observed after nine days, when all spermatogenetic cells have disappeared. The simple numerical relations between nuclear sizes are a strong evidence in favor of polyploidy, although the exact interpretation of these facts awaits further research.[33]

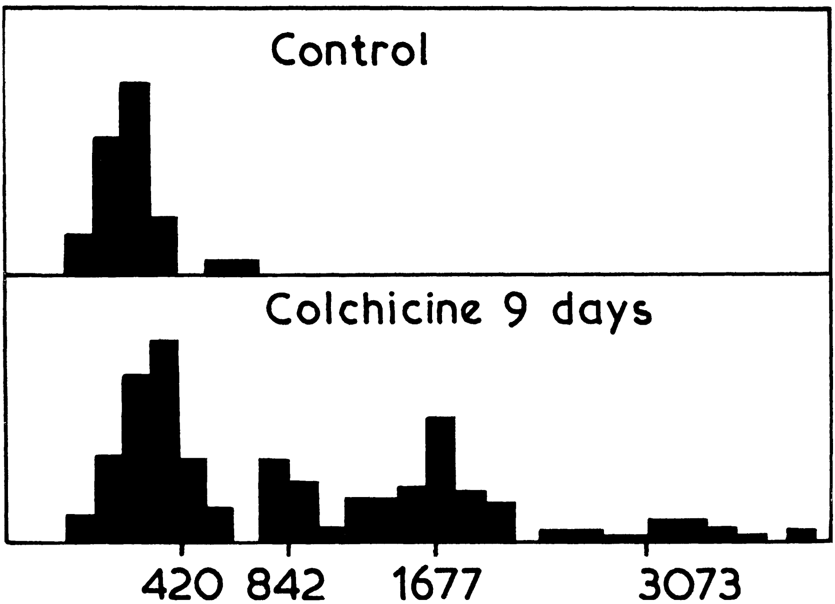

Fig. 8.1—Action of a prolonged treatment by colchicine on the nuclear diameters of the spermatids, expressed in conventional units, in **Triatoma infestans.** Several categories of polyploid nuclei with diameters in the relation 2,4,8,16. (After Schreiber and Pellegrino[33])

In Chapter 16, a technique of inducing polyploidy in vertebrates will be discussed. This involves using sperm treated with colchicine. It should be mentioned here that the alkaloid has not been reported to affect adult spermatozoa.[12, 13]

8.2: Colchicine-induced Malformations

The artificial production of embryonic monstrosities has received a great impetus from the work of Ancel and Lallemand.[1, 2, 21] This was initiated around 1937, and, together with the use of other chemicals, has opened a new field in developmental research. A detailed survey of this is to be found in Ancel's recent book, *La Chimiotératogenèse*.[1]

Through a small opening in a chick's egg, a minute quantity of a solution of colchicine in saline is introduced. The embryo is observed, to make sure that no abnormalities exist at the start of the experiment. The opening is closed and the egg hatched in an incubator.

One of the most striking results was the production of a malformation which had been described in calves by Gurtl (1832) and called *schistosomus reflexus*. This is a peculiar type of celosomy, that is, a total hernia of all the abdominal and thoracic viscera, resulting from an absence of the anterior body wall. Lesbre, in 1927, used the term *strophosomy*, or body-turned-inside-out, for the rachis and tail are strongly bent backwards, the hind limbs located close to the back of the head (Fig. 8.2). Such a malformation had never been seen in chicks, and naturally aroused great interest in colchicine. Further testing of more than fifty substances, several of which induced various abnormalities of development, demonstrated that only *ricine* and *abrine* could initiate strophosomy.

Figure 8.3 shows the difference between the formation of celosomy, which is much more frequent, and strophosomy; the posterior bending of the caudal part of the spine plays a great part in the second type of anomaly. The colchicine treatment of the eggs must be done within a quite definite period. The optimal period is after 48 hours of incubation; before this time, or after 68 hours, it is ineffective. Only 5 hours after the introduction of colchicine into the shell, the embryo demonstrates an exaggerated forward flexion of the infracardiac region. Many of the embryos die at this moment. Some also display a dorsal flexion of the caudal extremity of the rachis; these are the ones which will eventually become strophosomic. This malformation does not disturb the formation of the embryonic organs, and the chicks are capable of living nearly until hatching, the longest observed duration being 19 days. A similar condition had been

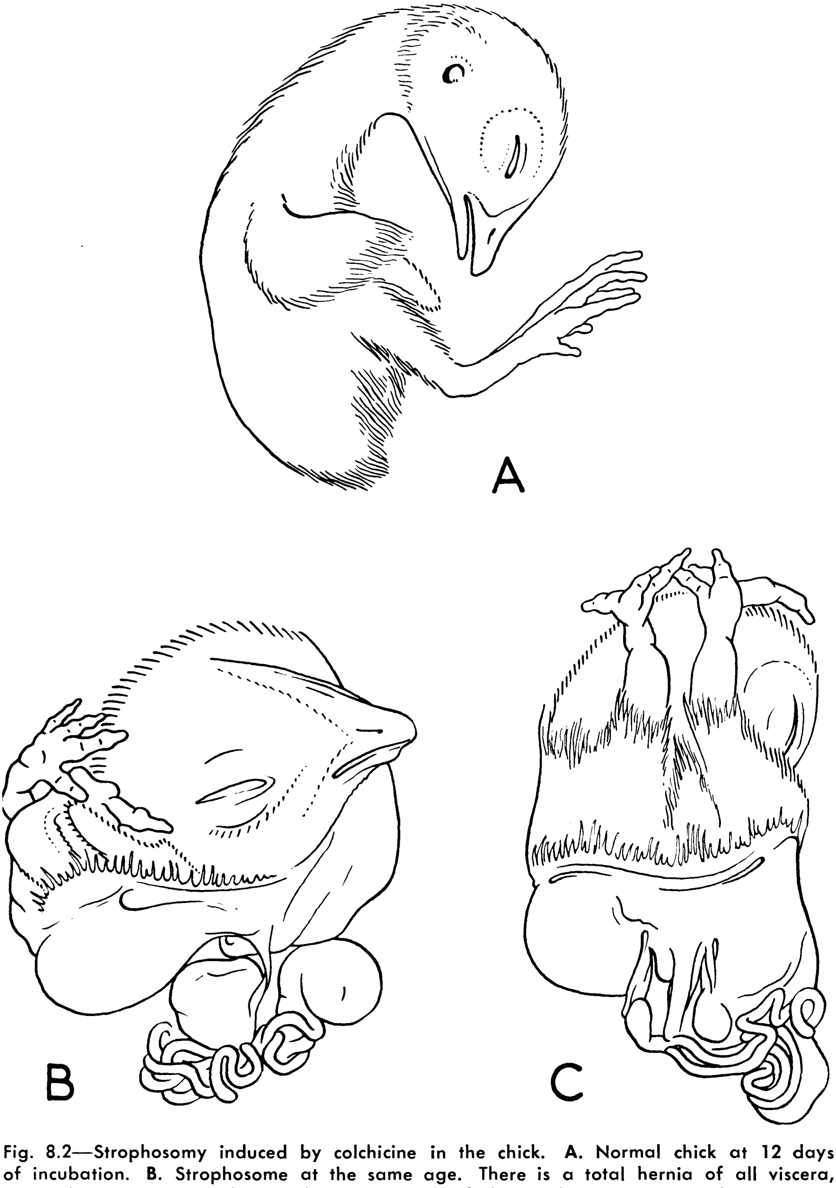

Fig. 8.2—Strophosomy induced by colchicine in the chick. **A.** Normal chick at 12 days of incubation. **B.** Strophosome at the same age. There is a total hernia of all viscera, no abdominal wall, and a backwards flexion of the hind limbs. **C.** Another strophosomic chick, after 13 days incubation. The animal is seen from the rear, the herniated viscera hang underneath, the legs here folded on the back. (After Lallemand[21])

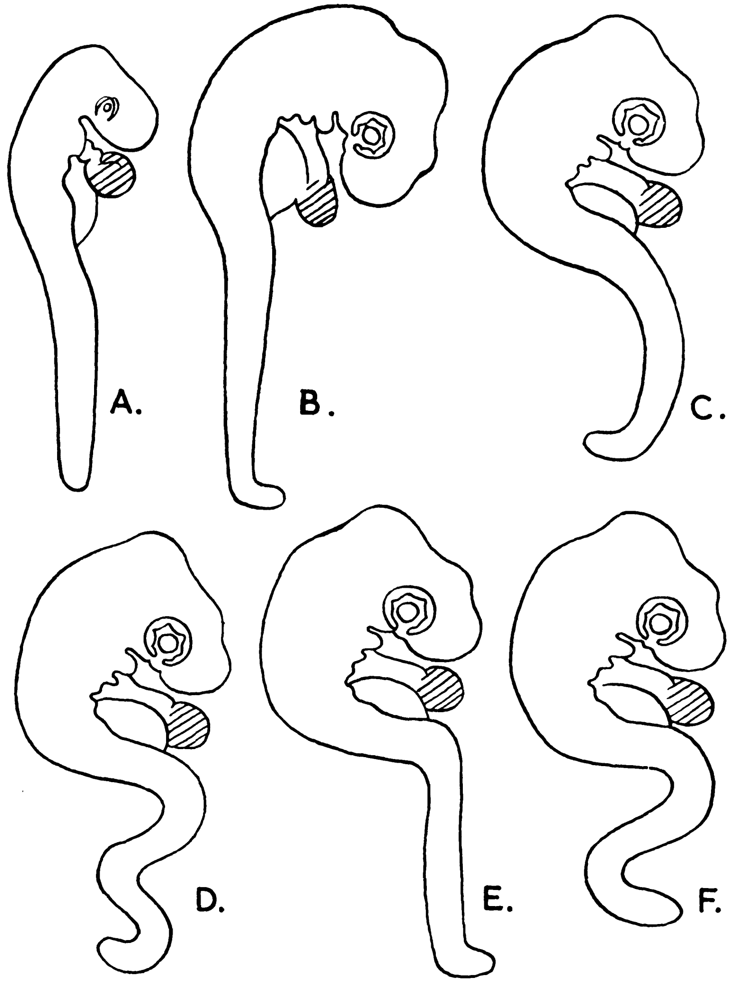

Fig. 8.3—Origin of strophosomy in chicks. Injection of colchicine in the eggs at 48 hours of incubation. A. Control at the time of injection. B. Control, incubated 72 hours. C, D. Colchicine-treated embryos, incubated 72 hours. These are future strophosomes, as indicated by the backward flexion of the tail. E, F. These chicks, similarly treated, will only develop celosomy. The tail is bent forward. (After Lallemand[21])

known to exist in calves, which may be born strophosomic after an intra-uterine growth of normal duration.

The caudal bending of the embryo appears quite important, and it is to be noted that pycnotic nuclei arising from arrested metaphases are to be found in this region, mainly in the nervous system and the surrounding tissues. Neither the chorda nor the intestinal epithelium shows evidence of cellular destruction.

The problem of the determination of strophosomy has been further studied by local applications of colchicine in agar strips.[10] In embryos with 25–28 somites, the region between the omphalomesenteric vessels and the hind limb is the most sensitive in regard to this malformation. Absence of tail and hypophalangism and absence of tail were also observed; these phenomena led to a study of colchicine on the expression of the anomaly, polydactyly.[10] In other animals, colchicine is also a teratogenic agent,[8] but the changes mentioned are of very different types, ranging from exogastrulation[7] to variations in pigmentation, cyclopean eyes, abnormal blood formation, and disturbances of body flexures.[39] In the frog, many of the reported anomalies[42, 43] could also be initiated by X-rays, a fact strongly suggesting their relation to mitotic disturbances.

One other result is worth mentioning. Local application of a 1:7000 solution of colchicine on the posterior limb of *Xenopus* larvae resulted in a decrease in the number of toes.[5] With increasing effects all but the fourth toe disappeared during development. This is paralleled by no other type of regressive evolution of toes in vertebrates.

8.3: A Tool for the Study of Embryonic Growth

The use of colchicine for the detection of zones of maximal growth and of growth stimulation or inhibition will be discussed at length in Chapter 9. The "colchicine method" is fundamentally based on the observed increase in metaphases, arrested because of the absence of spindle, in growing tissues. Mitotic multiplication of cells is made more visible. Some of the difficulties of this method in adult animals will be discussed in Chapter 9. It is evident from all that has been written in this chapter, that in embryonic growth the complexity of the changes brought about by colchicine is considerable. Not only does the alkaloid inhibit mitoses, it may also completely alter the normal course of growth. Only a few experiments yield facts that are simple to interpret.

For instance, in chick embryos treated at the forty-second hour of development with dilute solutions of colchicine, there could be observed, 24 hours later, an "overproduction of cells." [28] The amount of neural tissue appeared to be increased, and several neural folds were to be seen, even in animals where the number of arrested mitoses did not appear to be great. These facts were considered as good evidence of mitotic stimulation and increased neuralization, that is to say, a colchicine-induced malformation. Chicks with spina bifida have been found in some experiments.[1] The number of mitoses seemed considerable to the author who observed for the first time these neural changes, but no accurate quantitative counting was done, nor, in fact, could have been properly done because of the malformation itself. It has also been suggested that the apparent increase in neural tissue was merely the consequence of abnormal cellular migrations, not of modified mitotic activity.[45]

Analysis of patterns of embryonic growth is made difficult by many facts. One is the varying sensitivity of tissues and stages of development. In *Molge palmata* Schneid., the zones of highest mitotic activity are the most sensitive to colchicine;[35] in other regions, the same concentration may yet enable mitosis to recover and to proceed to telophase through star and incomplete star metaphase. In *Discoglossus pictus* Orth., some periods of growth are very sensitive to the mitotic arresting activity of colchicine. The fifth day, corresponding to the "primary metamorphosis," when swimming is initiated, is one of these periods. In *Discoglossis*, *Rana*, and *Xenopus*, the metamorphosis is a period of increased sensitivity. The regions of the embryos where the mitoses are the most numerous are, rather naturally, the most rapidly altered by colchicine. Instances are the nervous system, the olfactory bud, and the germinative region of the eyes.[35]

These carefully studied facts do not leave much to say about papers which attempted to detect zones of growth by colchicine, especially in amphibia,[15, 25] for the complexities of the problem were not properly understood at the time of their publication. Some facts emerge, however, from the literature on this subject and are worth mentioning, for they may be starting points for further work. In young mice, colchicine demonstrated that liver and pancreatic cells cease to divide at about 20 days after birth;[31] the mechanism which prevents any further division, except in regeneration (Chapter 9), is unknown. In mice also, ganglionic nerve cells have been found, by the use of colchicine, to divide until three weeks after birth.[20] Colchicine has also been used to bring about the death of the litter of pregnant mice,[19] and to induce the formation of tetra- and octoploid cells in embryos of the fish *Coregonus* when the eggs had been treated three hours with a 0.5 per cent solution. Hastening of the metamorphosis of *Rana fusca* tadpoles is also reported.[14]

The publications which have been reviewed in the last paragraph would seem to indicate that colchicine is of little, if any, use in the study of embryonic growth. However, it must be recalled that most of these results have been published during the early phases of colchicine research, before the proper techniques could have been designed. Two recent papers show that important facts can be made clear by using colchicine as a tool in embryos.[3]

In the first one, the problem was to assess the comparative mitotic activities of the embryonic megaloblasts (young red blood cells) of the chick embryo, and of the megaloblasts of human Addison-Biermer anemia (cf. Chapter 9). These cells resemble closely the embryonic ones, though their existence is an evidence of pathological growth related to vitamin B_{12}, or folic acid, deficiency. A dose of 0.015 mg. of colchicine in saline solution was found to arrest all mitoses in the young chick embryo. The number of mitoses found after four and eight hours was counted. This gives a precise idea of the proliferative activity of these cells. In chicks at the sixtieth hour of growth, eight hours after colchicine, the number of megaloblastic mitoses is increased more than tenfold; while in controls, 38.6 cells per thousand are in division; in treated chicks, eight hours after colchicine, the figure reaches 457.9. This increase is markedly greater than that found in the bone marrow of Biermer anemia patients. However, the technique being different, the comparison is not quite valid. What is more interesting from the viewpoint of embryological growth, is that the megaloblasts are demonstrated to divide more than the undifferentiated connective cells from which they originate.

A detailed study of the relation between differentiation of the red blood cells and cell division in the chick embryo at different stages of growth has clearly indicated a decrease in mitotic activity as soon as hemoglobin is synthesized. Colchicine has been a remarkable tool for the precise study of this problem.[3] No doubt, it will not be the last contribution in a field open to many types of investigation (cf. Chapter 9).

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