The Effect of a Water-soluble Carcinogen on the Early Development of the Frog

by

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Introduction

Experimental carcinogenesis, or the experimental production of tumors in animals, is a relatively new field of research. Its beginning dates from 1915 when the Japanese workers Yamagiwa and Itchikawa first succeeded in producing papillomas and true malignant growths on the ears of rabbits after continued application of tar. This important observation at once raised the question as to the nature of the component or components in tar which were responsible for its carcinogenicity.

The English workers, Kennaway, Cook and their associates, attacking the problem with this question in mind, finally succeeded in isolating 3,4-Benzpyrene which is now recognized as the active agent in coal tar. In the course of that work they were the first (Kennaway, '30; Kennaway and Heiger, '30) to demonstrate that certain pure hydrocarbons had the capacity to induce tumors in animals. This was confirmed by Cook et al ('32) with particular reference to purified 1,2,5,6-Dibenzanthracene.

Since that time work with chemical carcinogens has been extensive. (See comprehensive reviews by Cook et al, '37; Cook and Kennaway, '38 and '40). In general it has followed three main lines of attack: 1. The application of carcinogens to tissue cultures and to animals in an effort

to study the mechanism of the transformation of normal to malignant cells; 2. systematic synthesis and biological testing of new substances in an attempt to find some correlation, if possible, between chemical structure and carcinogenic properties (Fieser, '38 and others); and 3. the utilization of carcinogens as a means of production of standard known tumor types which can then be used for the study of the biology of the tumor cell.

The present work falls in the first of these three groups. In brief, we have reared frogs' eggs in solutions of a water-soluble carcinogen, the hope being that we could observe changes in the living cells which it is difficult to do in work with larger animals such as mice. Our procedure also appeared to offer some possibilities not available in tissue culture work or in work with small free-living organisms. Specifically, in the frog embryo certain fundamental developmental processes are taking place, and thus opportunity is afforded to observe the effect of the carcinogen on an actively growing and differentiating system.

Materials and Methods

General :

The frogs used were all Rana pipiens. They were collected from the Northern Lake Champlain region and kept in the laboratory in tanks with running water at 4 to 6° C. Ovulation was induced by pituitary injection (Rugh '34 and '37) and the eggs were artificially fertilized. As soon as the eggs had rotated (approximately one-half hour after fertilization) they were cut apart, checked over, mixed and counted out into Syracuse dishes. All excess liquid was then removed and the eggs were quickly put into finger-bowls containing the appropriate solutions. 10% Ringers solution was used throughout for the controls and for the dilution of all experimental solutions.

In the first nine experiments (see appendix I for table of experiments) dishes were kept on a laboratory table. However room temperature was found to fluctuate to such a degree that uniform results were impossible. Therefore a water-bath thermostat was set up with a mercury-toluol thermoregulator (Bridges '32) and a vacuum-tube relay. By this means it was possible to maintain a temperature of 19.6 ± .01° C. and all animals

from experiment 10 onwards were raised at this temperature.

Growth measurements were made by means of an "embryometer". (See appendix II).

Stages in development were identified according to Shumway's tables for the normal development of Rana pipiens (Shumway '40).

Reflexes were tested in several experiments taking criteria used by DuShane and Hutchinson ('41) for various physiological stages. They are as follows:

NR - No response to repeated stimulation and pressure on myotomes.

NM - Non-motile or myotomic response. Slow contraction toward side stimulated followed by slow relaxation.

EF - Early flexure. Rapid reflex response; bending of body away from side stimulated.

Coil - Culmination of the early flexure response, attained when tail touches or passes head at height of response. May be followed by coil in opposite direction.

S-reaction - Wiggle superimposed on coil.

ES - Early swimming response; S-reaction results in forward motion of not more than 3 body lengths.

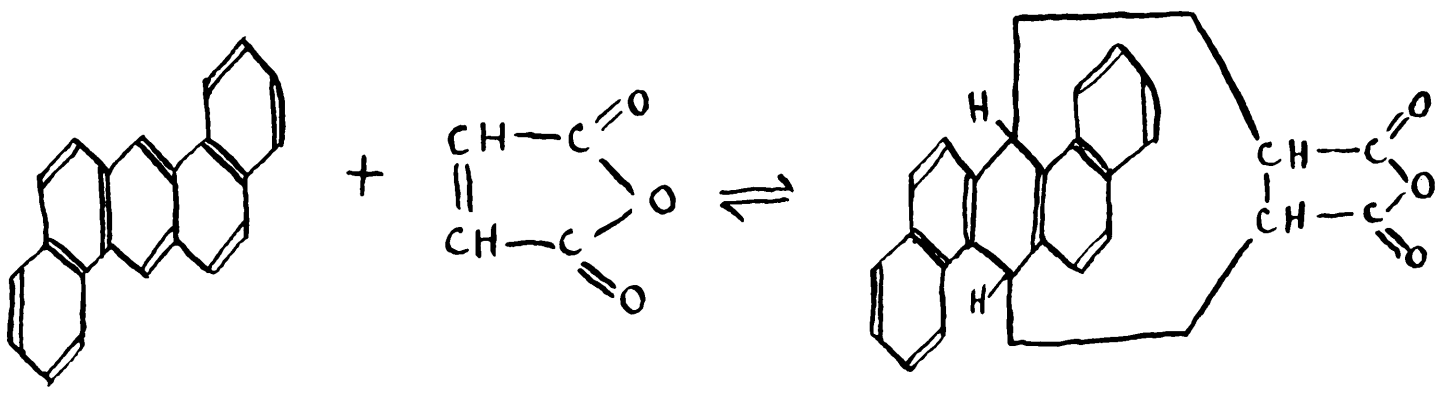
SS - Strong swimmers; forward motion of more than 3 but less than 10 body lengths.

LS - Late swimmers. Embryos which swim 10 or more lengths.

During the course of all experiments camera lucida drawings were made at a constant magnification of 13.2 x. When embryos were preserved, Heidenhain's "Susa" was used as the fixative and the tissues were run up through amyl acetate according to the method of Drury ('41) which prevents hardening of yolk material. Sections were stained with Feulgen and counter-stained with light green.

Chemical :

The carcinogen used in these experiments was a water-soluble derivative of 1,2,5,6-Dibenzanthracene. The parent hydrocarbon, first synthesized by Clar ('29) and later in a more pure form by Cook ('31), was obtained by us from the Eastman Kodak Company. It was then converted to 1,2,5,6-Dibenzanthracene-9,10-endo-α,β-succinic anhydride by reaction with maleic anhydride according to the improved method of Bachmann and Kloetzel ('38):—



The anhydride was then hydrolyzed to the corresponding dicarboxylic acid. The sodium salt of this acid is water-soluble only to a certain extent in the usual (cis) form. However the trans form was found by Warren ('39) to be more readily soluble and therefore our compound was converted to its sterioisomeric form according to Warren's method. (This work was all done by Mr. R. Schissler, Department of Chemistry, McGill). The final compound, 1,2,5,6-Dibenzanthracene-9,10-endo-α,β-succinic acid (trans) was pure white and had a melting point of 255-6° C. (corr.). It has been kept tightly closed in the ice-box and has shown no deterioration over a period of three years.

In making up solutions the free acid was converted to the Na-salt by heating the desired amount of DBSA * with 1/10 N NaOH (slightly in excess of the calculated amount needed) and distilled water (slightly in excess of the amount needed on the basis of 0.2% solubility of the

* Henceforth, for the sake of brevity, DBSA will be used to indicate the succinic acid derivative of dibenzanthracene (or its Na-salt), in contrast to DB, which will refer to the parent hydrocarbon, 1,2,5,6-Dibenzanthracene.

carcinogen.) After cooling, the solution was filtered and diluted with 10% Ringer's solution. The solutions showed no deterioration after being kept at room temperature for a period of months, but the 10mg/100 cc. concentration and to some extent the 4.26mg/100 cc. concentration showed a flocculent bluish precipitate (with consequent weakening of carcinogenic activity) after being kept in the ice-box for the same length of time. (Experiment 17).

Dosages were originally chosen on the basis of work reported by Creech ('40) (communicated by letter in '39). Using water-soluble DB-choleic acid in tissue culture, she found that a concentration of 0.01mg per cc. (equivalent to 0.0015 mg DB per cc. , which is equivalent to 0.213 mg. DBSA/ 100 cc.) caused significant increase in cell proliferation, while ten times that concentration resulted in retardation of cell growth. Our series of concentrations was accordingly set up to include these two dosages as well a one each below and above them: - 0.02; 0.213; 2.13; and 4.26 mg./100 cc. Subsequently we added a stronger concentration, 10 mg./100 cc., to the series.

It may be noted here that Lettinga ('37) found that as small a dose as 0.0125 mg. could produce tumors in mice

(4 out of 20 animals), and 0.5 mg. appeared to be a limiting amount beyond which there was neither decrease of latent period nor increase of tumor incidence.

Dobrovolskaia-Zavadskaia ('38) reported a single injection of 0.0025 mg. of DB to be capable of producing tumors in mice.

Levine and Bergmann ('40) induced tumors in mice by a single injection containing 0.0033 mg. DB and observed that repeated injections caused greater injury but no greater tumor production.

Calculating the weight of a mouse to be approximately 35 grams, and the water volume to be approximately 85%, the effective concentrations of the minimum doses above are found to be 0.04 mg./100 cc. (Lettinga), and 0.008 mg./100 cc. (Dobrovolskaia-Zavadskaia); and the maximum effective doses are 1.66 mg./100 cc. (Lettinga) and 0.011 mg./100 cc. (Levine).

Many workers have ofcourse induced tumors using much larger doses of DB, but these calculations serve to show - to the extent that work with mice and Amphibia can be compared - that not only the upper members of our series but the entire range of concentrations might be considered to be roughly within carcinogenically effective limits.

Special procedures for various control expeiments will be noted in the next section in connection with the specific experiment concerned.

Results

A table summarizing all experiments done, the number of eggs observed in each and other pertinent data, will be found in appendix I. For the sake of clarity, however, the results will be outlined here in the manner listed in the table of contents rather than in the order in which the experiments were originally numbered or carried out.

I. "BASIC" EXPERIMENTS

The eggs in all of these experiments (1-10 inclusive; 16; and 19;) were put in the various concentrations of the carcinogen 1 hour ± 5 minutes after fertilization. There was found to be no effect on rate of development during cleavage (Figures 1, 2, and 3). Similarly initiation of gastrulation took place in all groups of eggs at approximately the same time (Figure 4).

The first observable effect was a retardation which became evident during early yolk plug stage. Concentration 0.02 * caused no effect; 0.213 caused slight effect; but the three remaining concentrations, 2.13, 4.26, and 10.0

* All concentrations, unless otherwise stated, refer to milligrams per 100 cc.

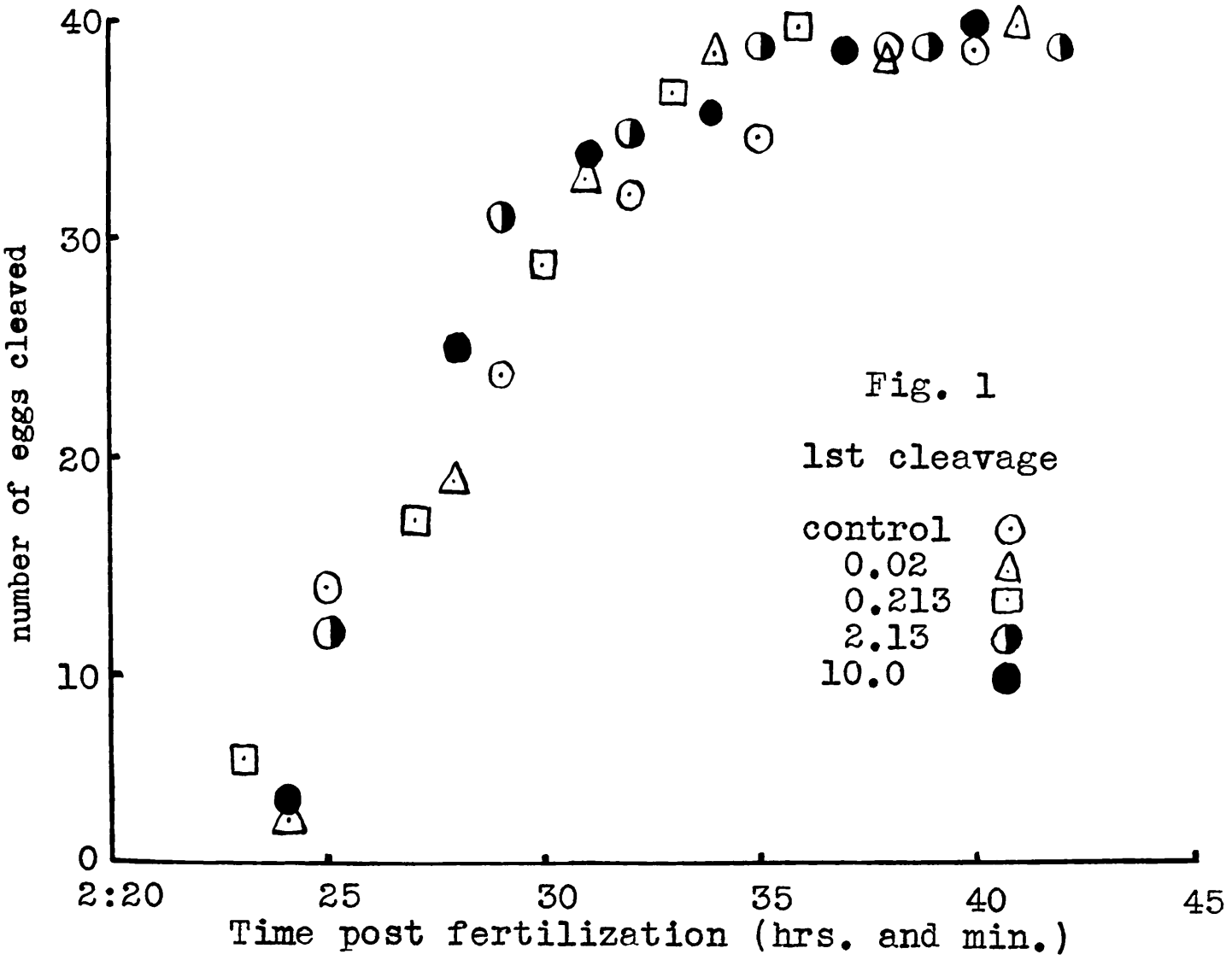


Fig. 1

1st cleavage

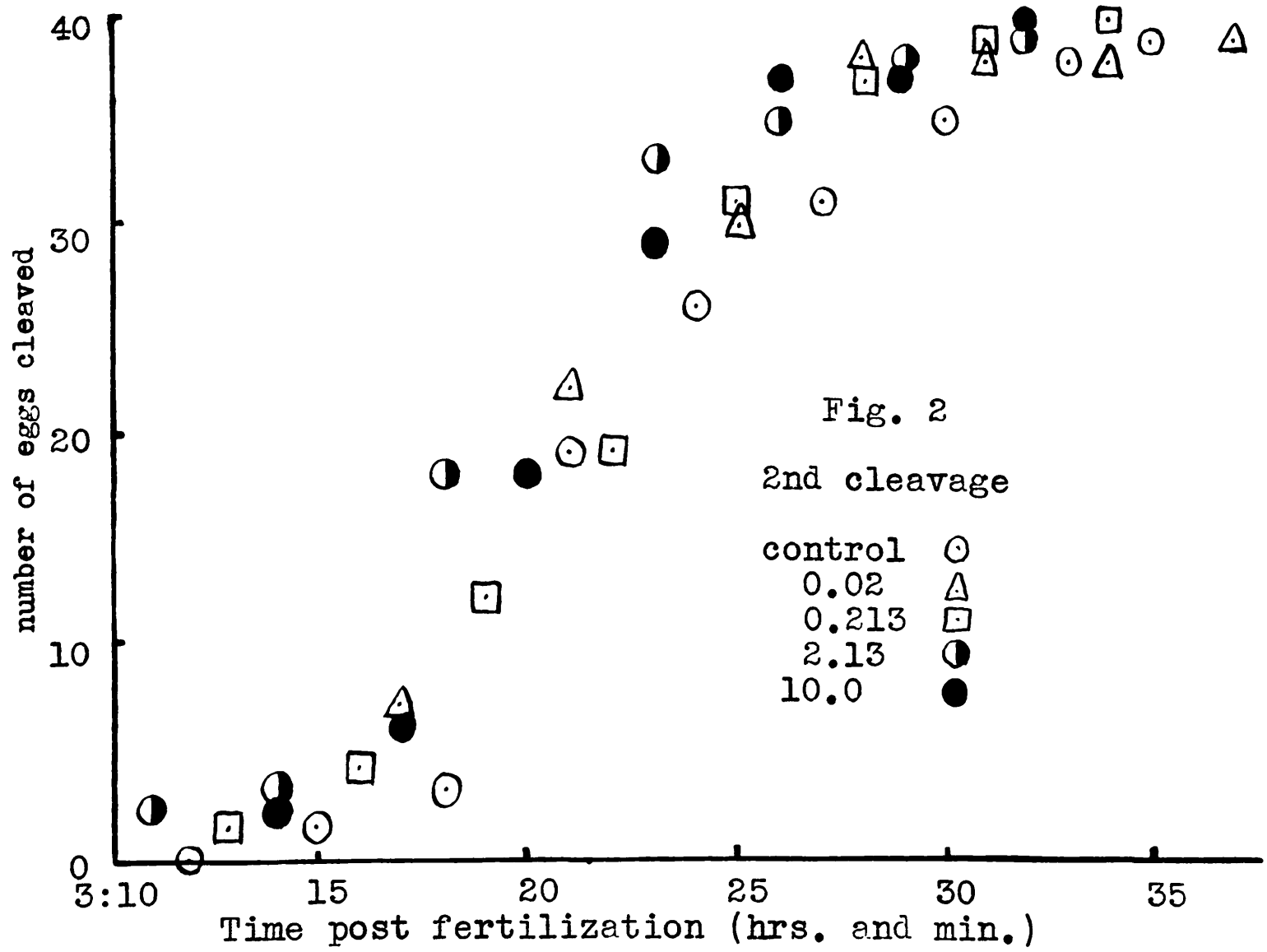


Fig. 2

2nd cleavage

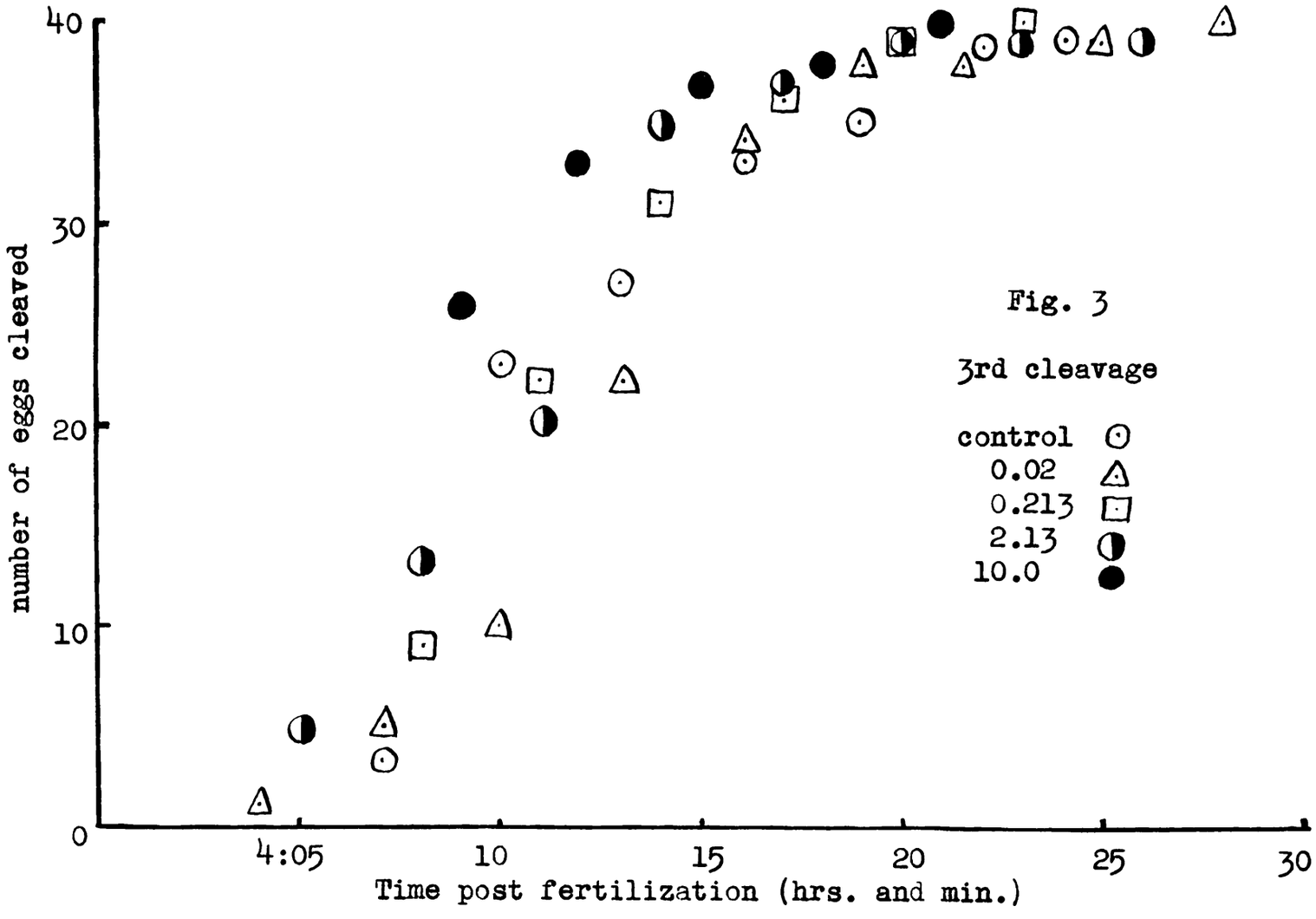


Fig. 3

3rd cleavage

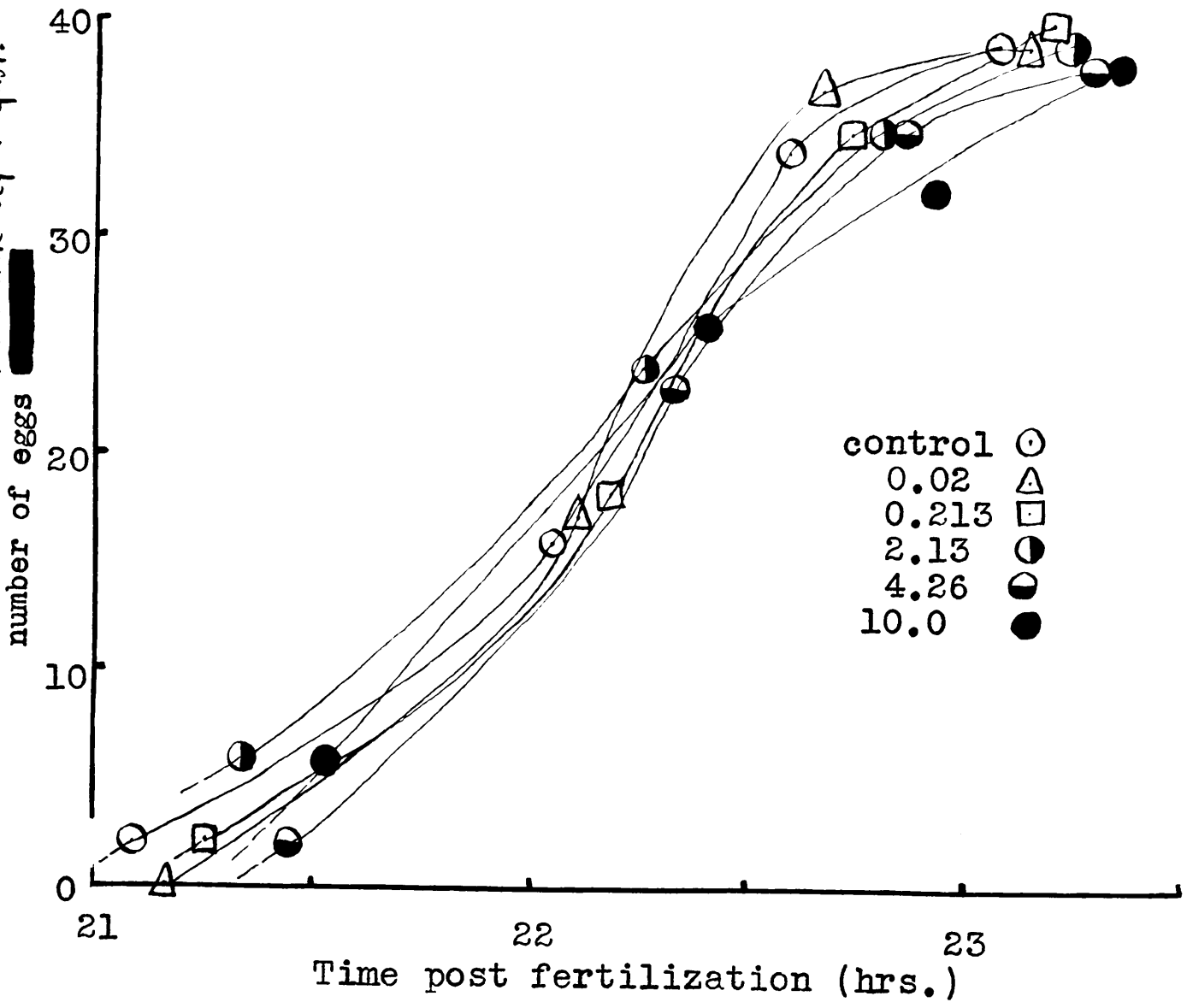


Fig. 4. Beginning of gastrulation.

definitely retarded gastrulation in the order given.

Closure of the blastopore took place at the same time in the controls and concentration 0.02; was sometimes but not always retarded in 0.213; definitely retarded in 2.13; long delayed in 4.26; and completely inhibited in 10.0. (Figure 5.).

When the controls were at approximately stage 14 (well-defined but wide apart neural folds), a new and striking development was observed, namely the uniform beginning of degeneration of the embryos in concentration 10.0. (A detailed description of the gross and histological appearance of this degeneration follows the present outline of the effect of the carcinogen on rate of development).

Within ten to fourteen hours after its first appearance at the lips of the blastopore, the degeneration had spread over the entire ectoderm. At the time of death (approximately stage 12-13½) the yolk plug was still visible in all cases, although sometimes it was almost obscured by the greatly swollen cells at its margin.

The eggs in the remaining dishes, meanwhile, continued to develop, showing retardation which was roughly proportional to the concentration of the carcinogen. The degree of

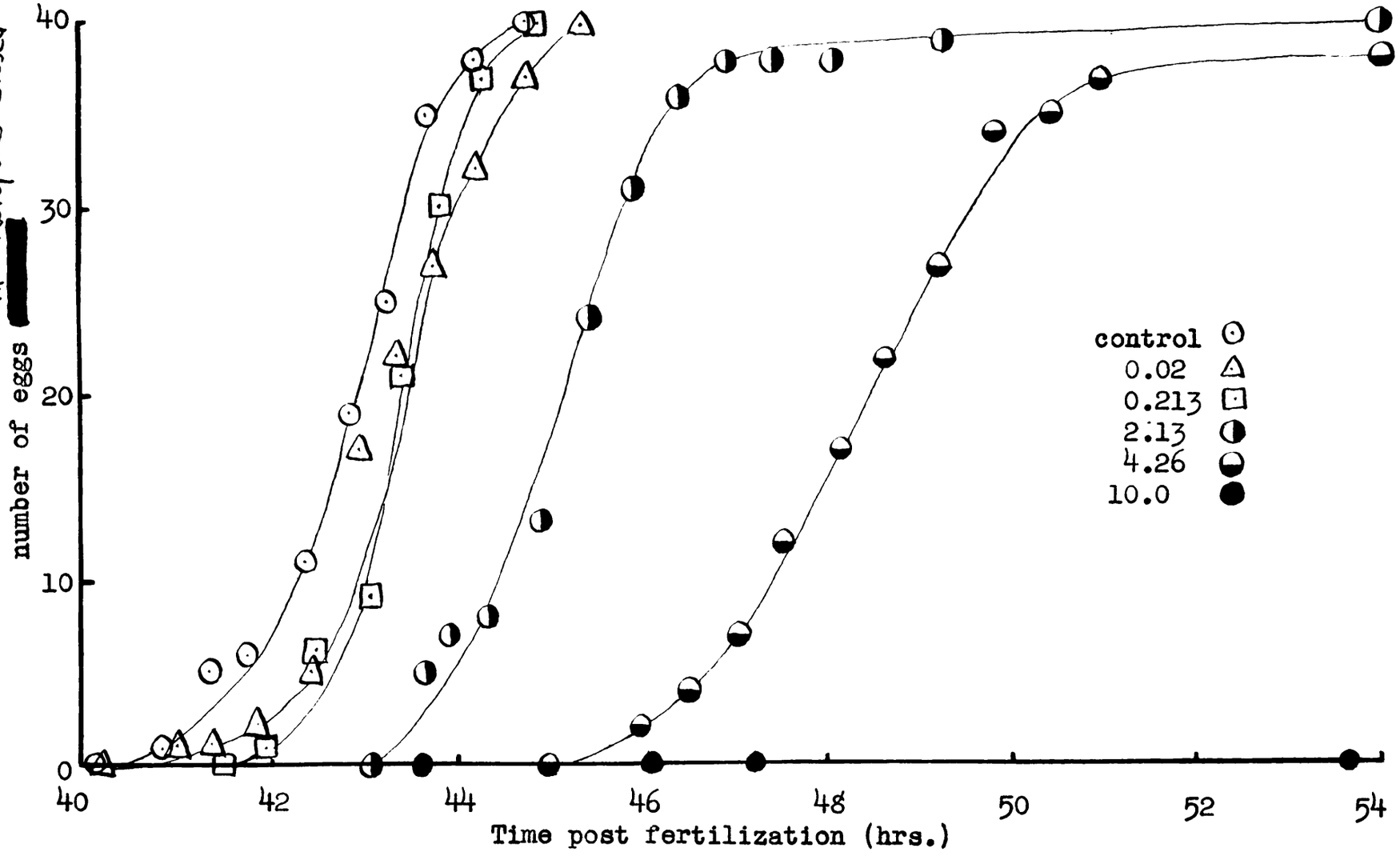


Figure 5. Closure of blastopore

retardation, as observed at the next well-defined developmental stage, - fusion of neural folds - is shown in figures 6a and 6b.

Effects at this stage, although similar in their general nature, varied somewhat as to details. In experiments 2 and 10, for example, (figure 6a) eggs in concentration 0.02 completed neural fold fusion slightly earlier than the controls, although in other experiments this acceleration effect was not observed and thus it is of doubtful significance. In some cases, for example experiment 10, concentration 2.13 retarded but did not inhibit neural fold fusion as was the case in experiment 16 (figure 6b). It is possible that such variations in results may be due to differences in vitality among different batches of eggs; or they might also be due to slight variations in the different batches of solutions, although weighings were done very carefully on a balance accurate to ± 0.1 mg.

When the controls reached stage 15½-16 (neural folds fused or fusing) degeneration began among the embryos in concentration 4.26. The character of the degeneration was histologically exactly the same as that which will be described below for the eggs in concentration 10.0.

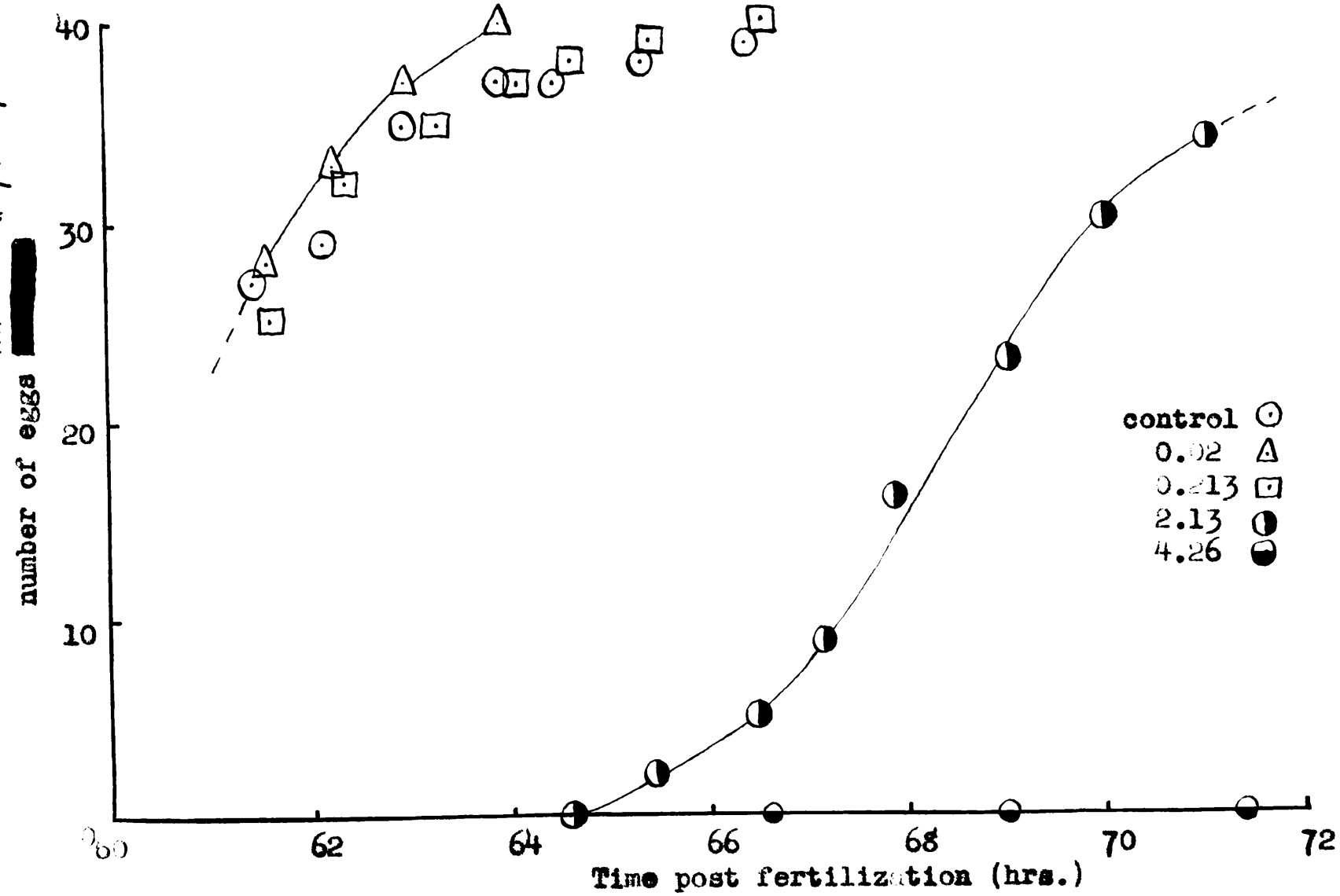


Figure 6-a. Neural fold fusion. (Exp. 10).

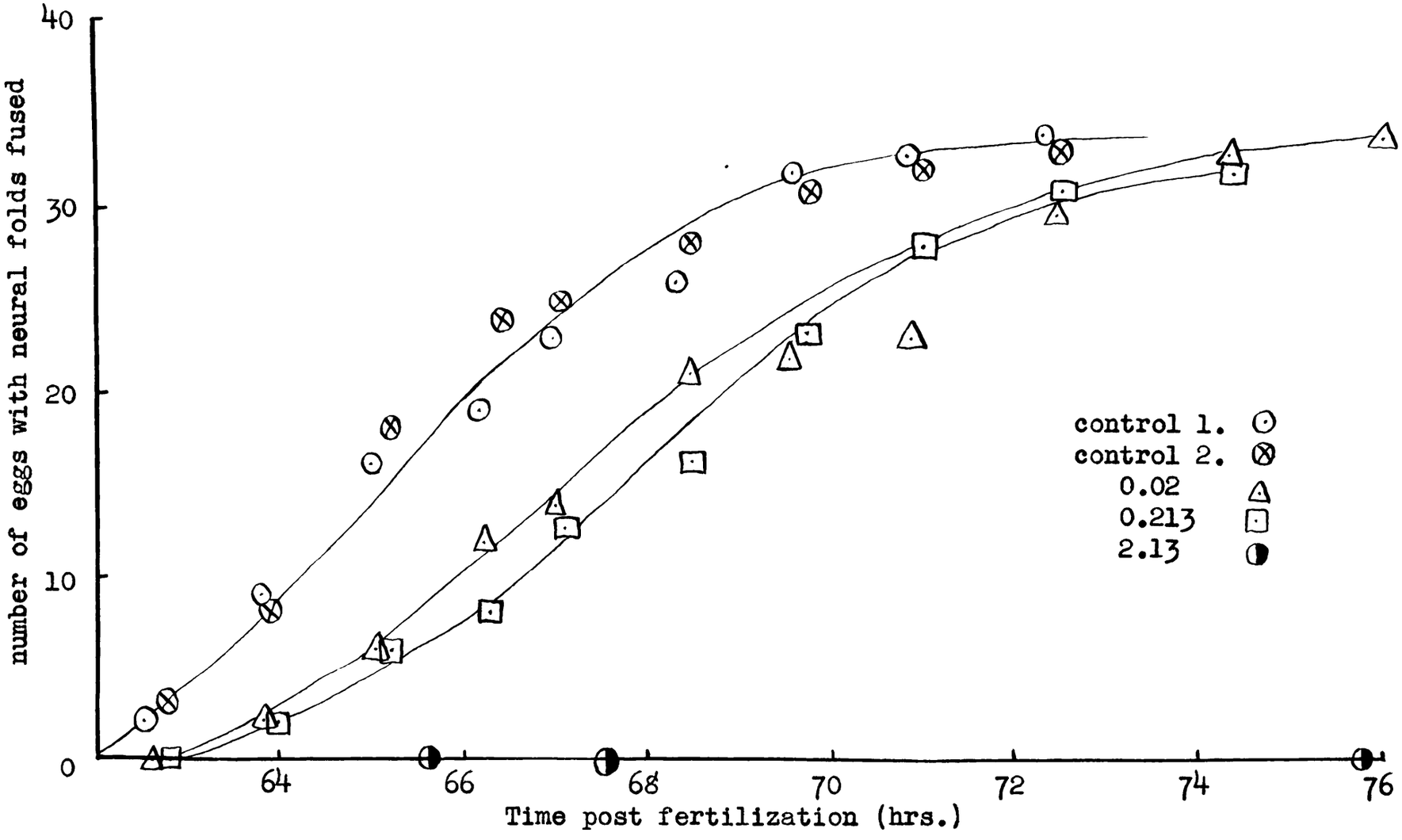


Figure 6-b. Neural fold fusion. (Exp. 16).

However in this case the effect was specifically restricted to the neural fold cells, degeneration spreading over the body ectoderm only secondarily after complete destruction of the neural fold tissue had taken place.

The embryos in concentration 2.13, as has been mentioned, did not react uniformly in all experiments, suggesting perhaps that this concentration is in some respects a threshhold value under the conditions used by us.

In experiment 16 neural fold fusion took place at the posterior and anterior dorsal regions, but a small area approximately over the hind-brain remained open. It was in this exposed portion of the neural groove that degeneration began. Development continued, including the formation of a well defined tail-bud, but the neural folds never closed completely and degeneration spread backward along the neural groove and neural folds. Later the lateral and ventral body ectoderm became "pebbly" in appearance (this condition often preceeded degeneration of body ectoderm), the cells became pale and swollen and sloughed off, and the animals died without ever hatching or exhibiting any flexure movements.

In experiment 10, on the other hand, the neural folds of the 2.13 animals fused completely and the animals hatched. However they were definitely retarded relative to the controls. (This retardation is evident in figure 7, which shows post-neurula growth curves for this experiment; and in figure 8, a and b, which shows sections of control and 2.13 animals of the same age at comparable levels).

Furthermore the 2.13 animals in this experiment did not respond to mechanical stimulation even after they had passed the morphological stage at which such responses should become evident. This fact suggested the possibilities that either there was differential retardation of presumptive neural tissue, or that some injury to the presumptive neural tissue had occurred although it had never been grossly evident. Further evidence in support of such hypotheses was found in the fact that the animals in concentration 2.13 succumbed about twice as quickly as did the controls when they were anesthetized for the purpose of making growth measurements. Some of these 2 .13 animals ultimately showed degeneration of central nervous system or of tail tissue.

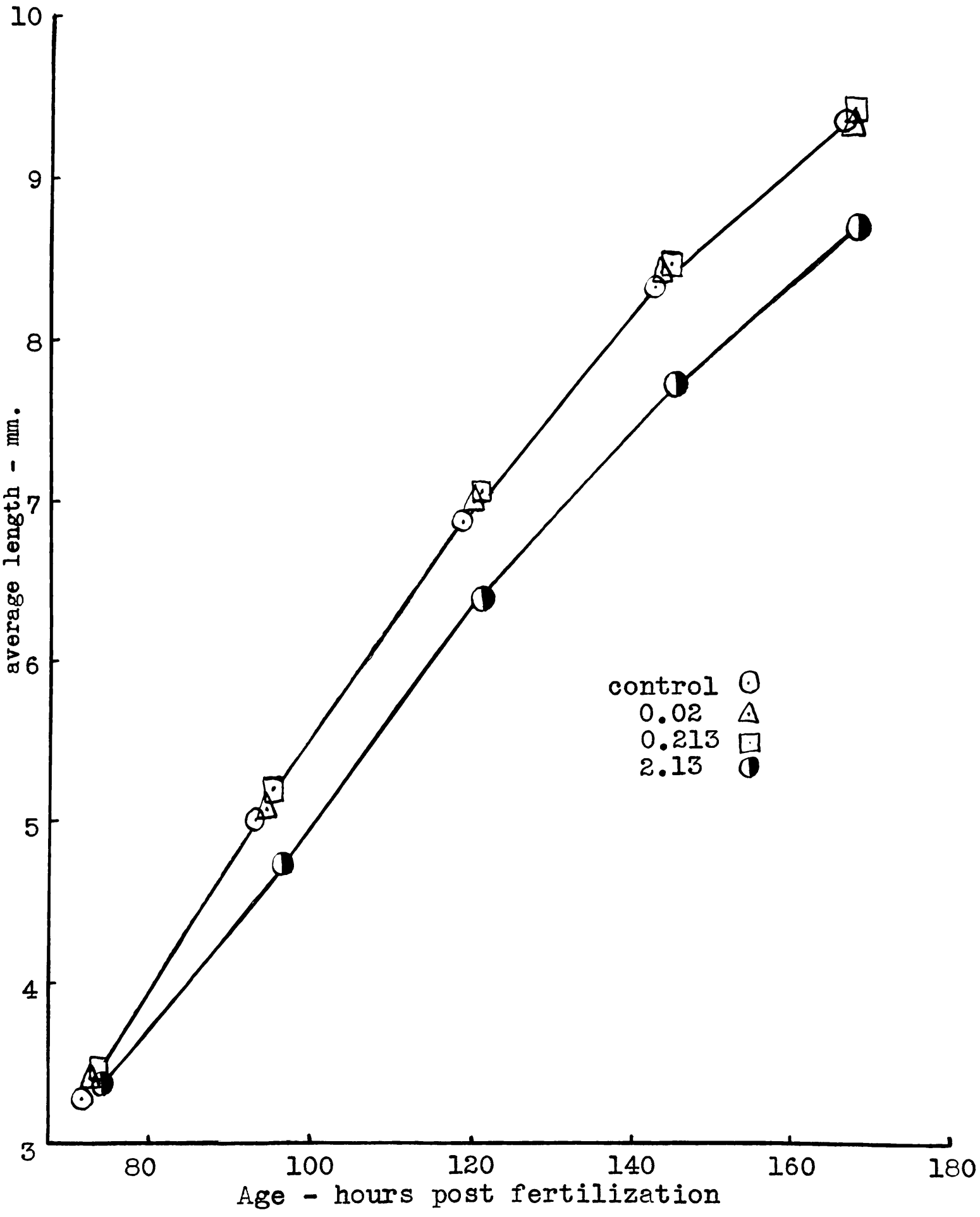
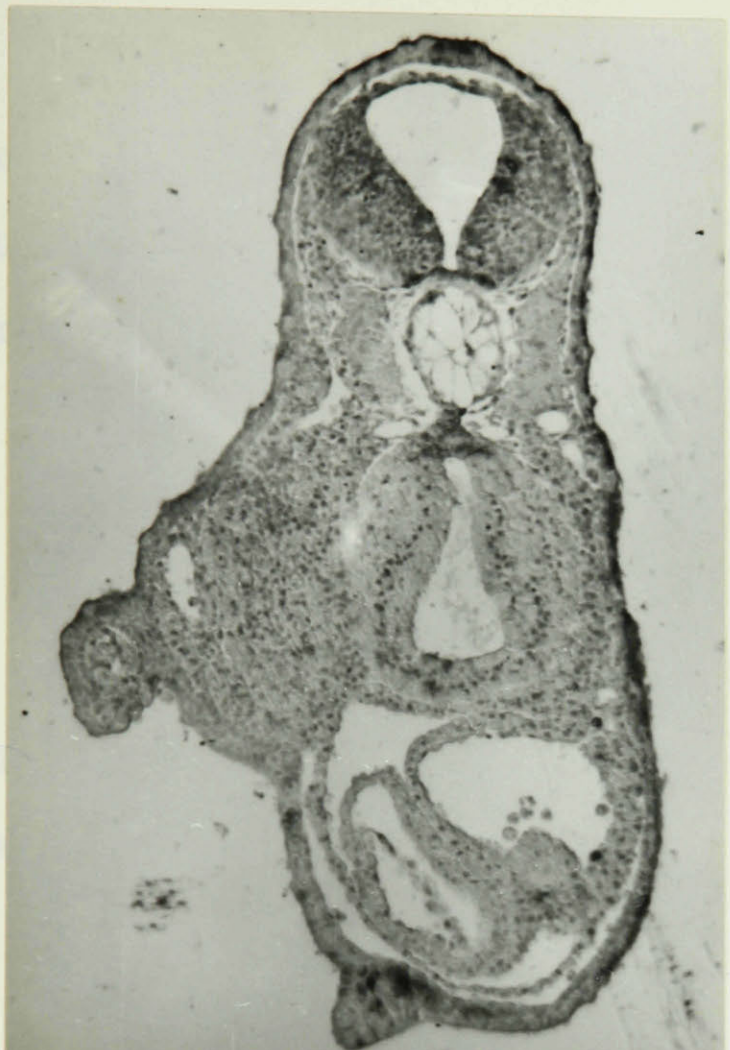


Fig. 7. Post neurula growth (Exp. 10).



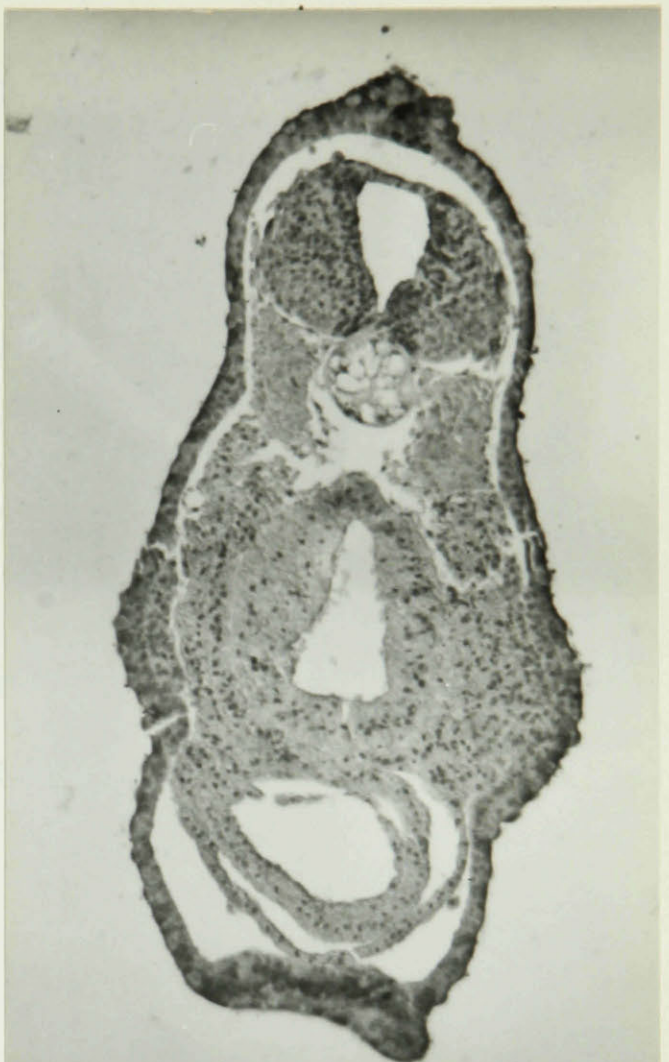


Fig. 8-a

Fig, 8-b

Figure 8-a shows a control embryo (stage 19; heart beat, muscular movement). The section is through the maximum diameter of the heart.

Figure 8-b shows an embryo from concentration 2.13, of the same age and from the same experiment as the embryo shown in 8-a. The experimental embryo is at approximately stage 18.5 and shows neither heart beat nor muscular movement, although movement should become evident at stage 18.

It may be seen that in the 2.13 animal the heart and gills are less well developed, and the notochord is less vacuolated than in the control. The neural fold fusion, although complete, is not smooth and normal.

(mag. app. 67 x).

Finally, in experiment 2, the 2.13 concentration animals, although definitely retarded relative to the controls, developed into active and to all appearances normal tadpoles. No sign of degeneration was ever seen.

Figure 9 is a general summary of the observations from all these experiments, in terms of camera lucida drawings.

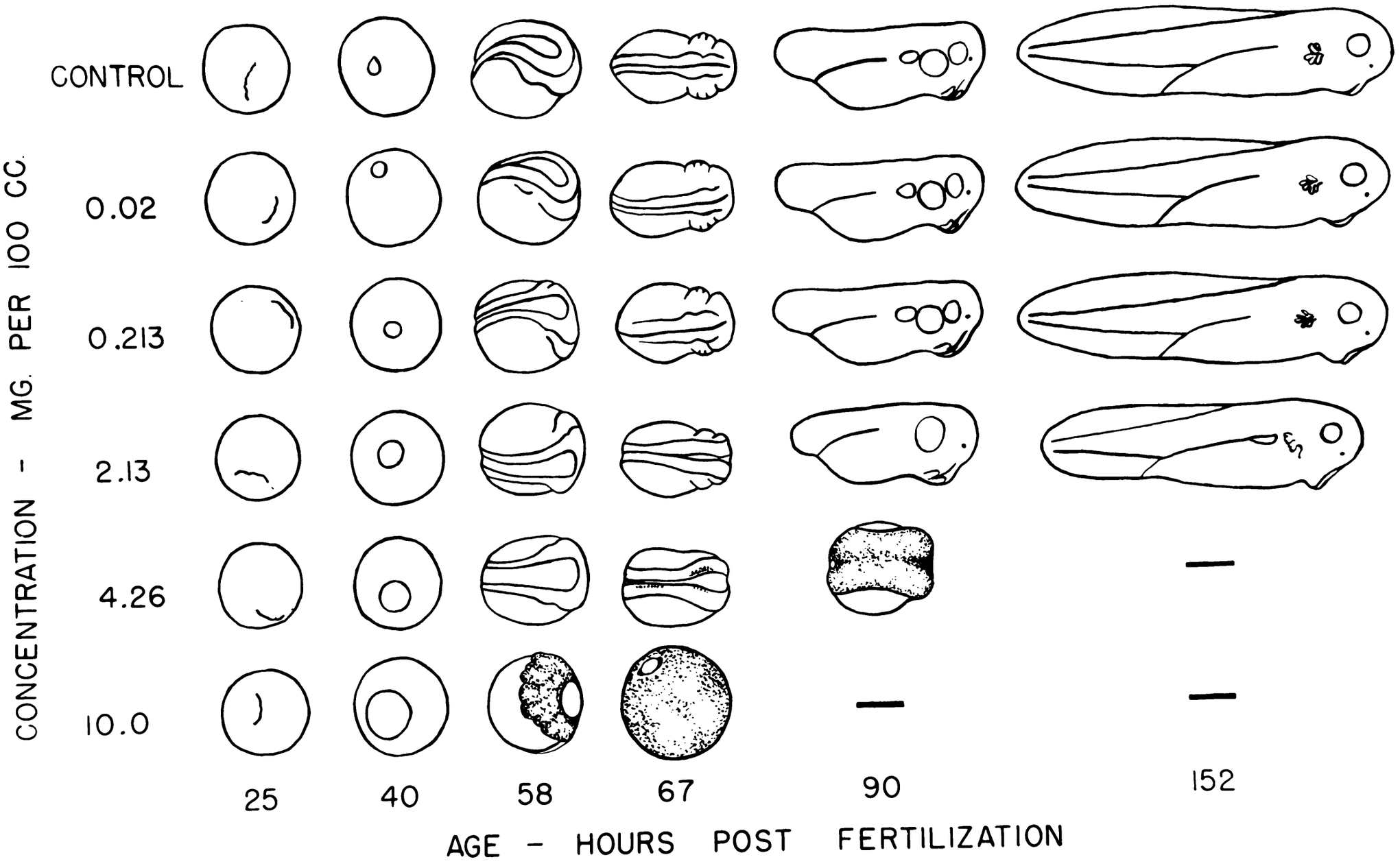


Figure 9. Summary of the effects of a water-soluble derivative of 1,2,5,6-dibenzanthracene on the early development of the frog. (Mag. app. 8 x).

A word may be added in regard to the gross and histological appearance of the degenerative changes which were observed. In concentration 10.0 degeneration always began at the lips of the blastopore and was usually preceeded by pigment recession in that region. In the earliest stages of degeneration it was often possible to observe the first single degenerated cell by its lack of pigment and by the fact that part or all of it appeared to be swollen and bulging out beyond the normal contour maintained by the neighboring cells. This earliest degenerative change is seen in section in figure 10-a, and in a semi-diagrammatic drawing of the corresponding living egg in figure 10-b.

The degeneration spread rapidly forming a well defined "collar" of pale degenerated cells around the persistent yolk plug (figure 11, a and b).

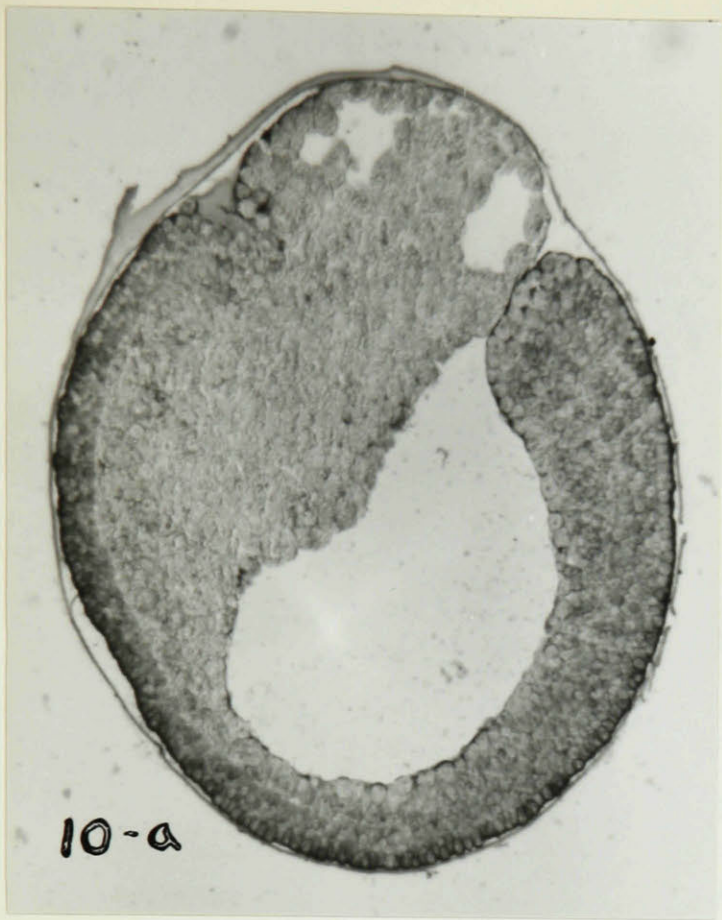
Histologically the following changes appear to take place:

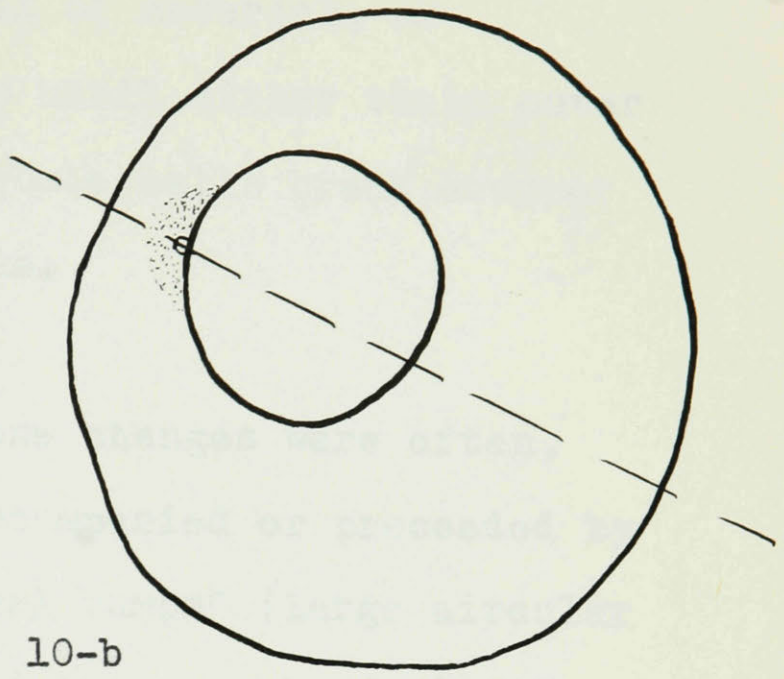
1) The outer ends of superficial cells become rounded and protrude.

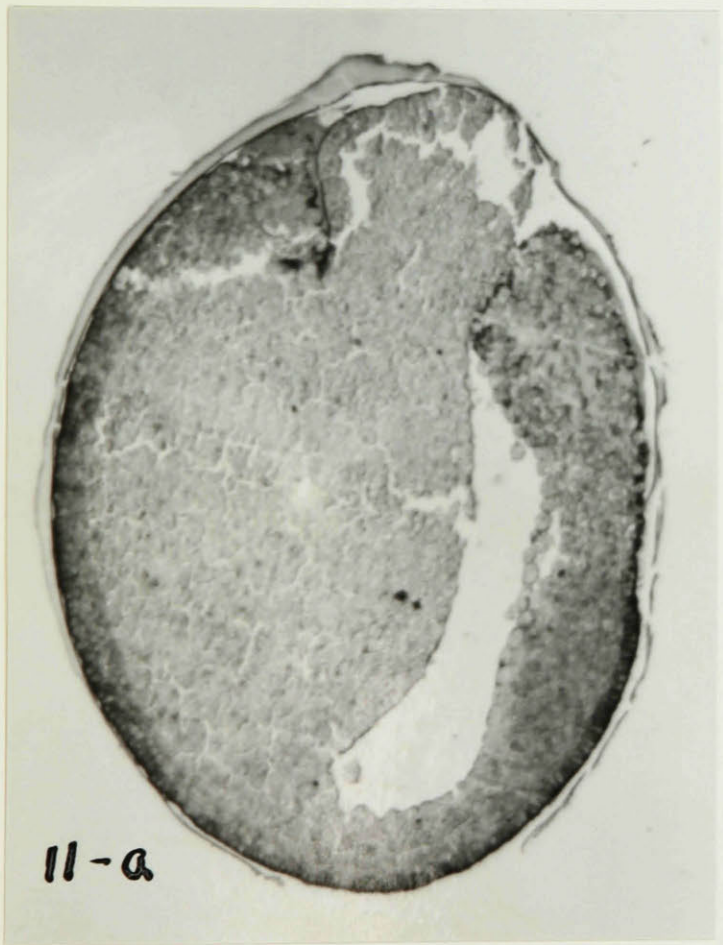
2) Pigment retreats from the surface of the cells.

3) Nuclei, in some cases, become pycnotic.

4) Either cytolysis occurs and cell fragments







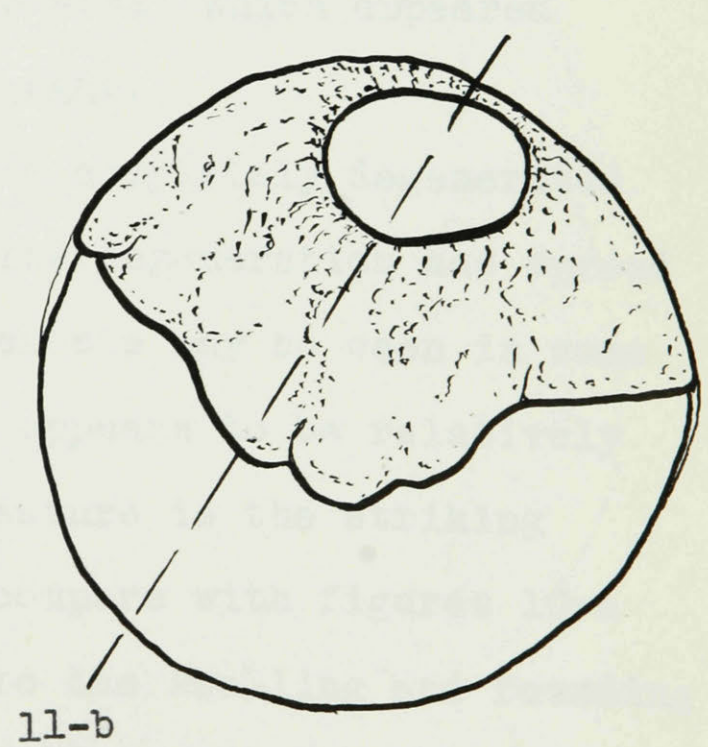


Fig. 10. (Conc. 10.0). Earliest stage of degeneration. A single, pale, swollen cell can be seen at the ventral lip of the blastopore. The dorsal lip is normal. Stippling in 10-b indicates area of pigment recession.

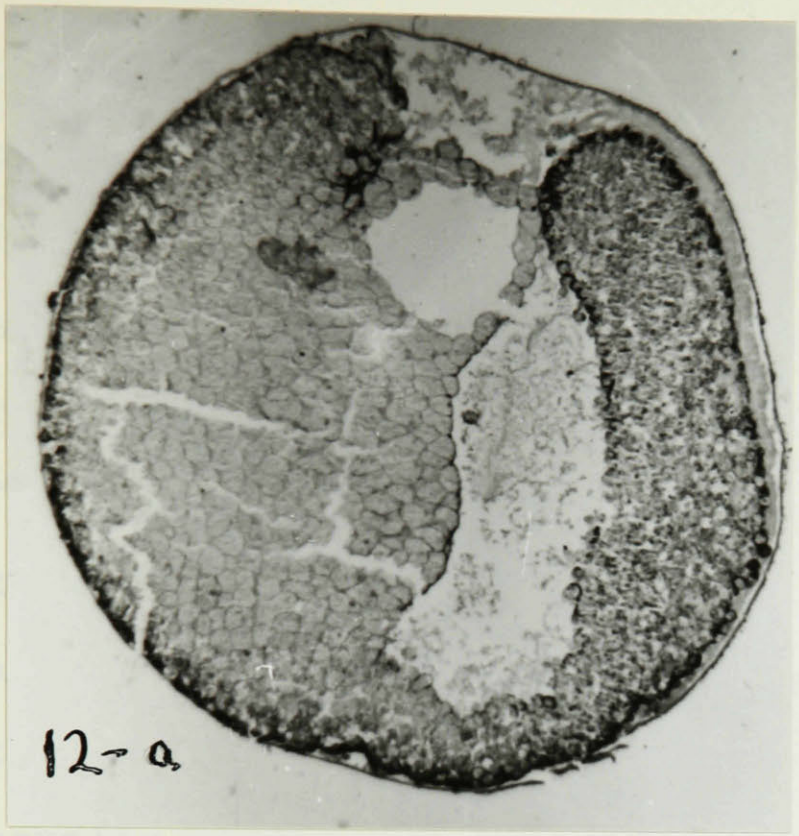
Fig. 11. (Conc. 10.0). "Collar" of degenerated cells. (mag. 39 x).

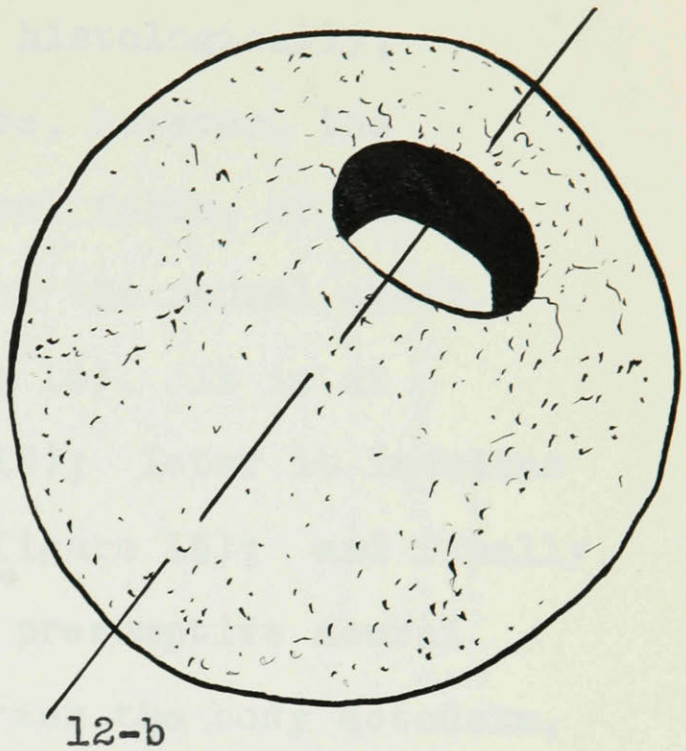
are seen in an amorphous matrix of material, or

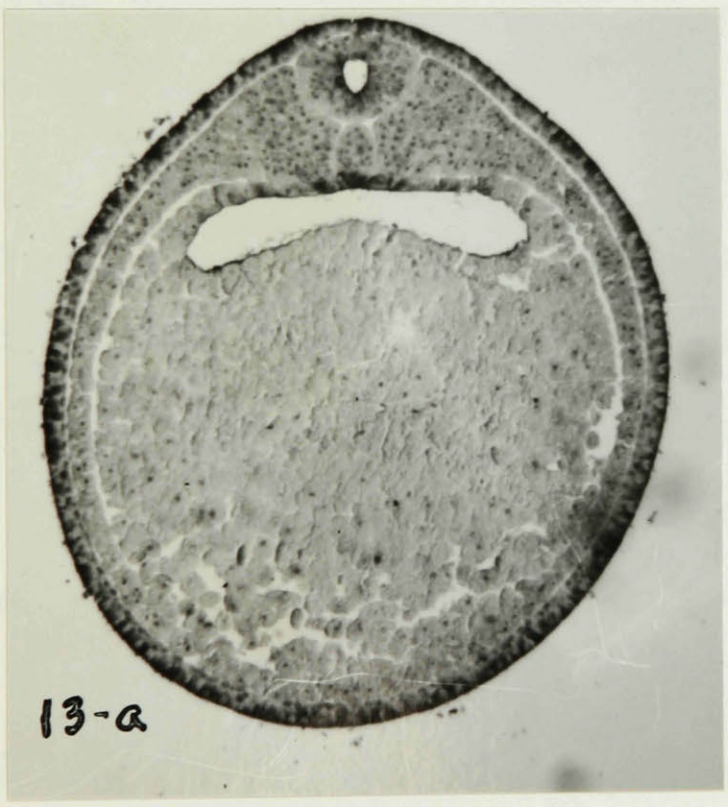
5) the cells swell up until either their outer ends are pinched off, or the whole cells break away or are ejected from the epithelium.

In concentration 10.0 these changes were often, but not necessarily always, accompanied or preceeded by the appearance of either "dorsal bumps" (large circular constrictions on the surface of the egg always in a constant position relative to the yolk plug); or small non-cellular greyish "exovates" which appeared anywhere on the surface of the eggs.

Figure 12, a and b, shows a completely degenerated egg from concentration 10.0. The degeneration has spread over the entire surface and pycnosis may be seen in some regions, but the yolk material appears to be relatively unaffected. One interesting feature is the striking enlargement of the whole egg (compare with figures 10-a and 11-a) due perhaps in part to the swelling and rounding up of individual cells. In the living or just-died egg such swelling, on the part of cells at the lips of the blastopore, often results in the partial or complete obscuring of the yolk plug. Figure 13, a and b, shows the stage of development of the controls at approximately the time that degeneration becomes complete in the 10.0's .







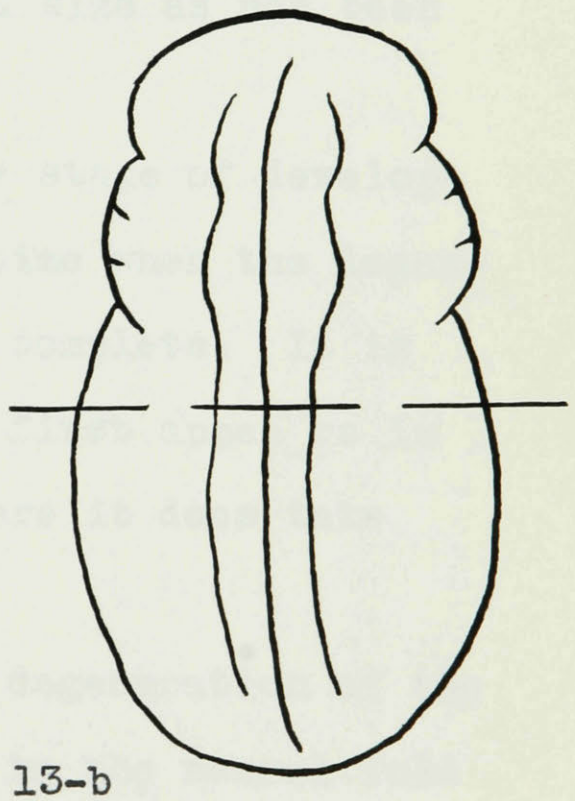


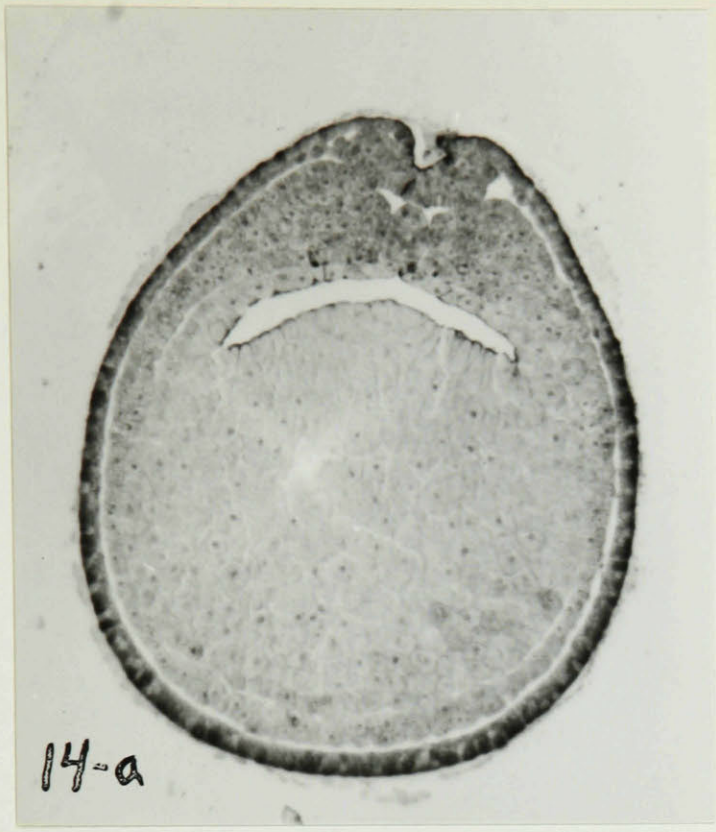
Fig. 12. (Conc. 10.0). Complete degeneration. Note enlargement of entire egg. The apparent sinking-in of the yolk plug is probably due in part to swelling of cells at the lips of the blastopore. Pycnotic nuclei and rounded-up superficial cells may be seen.

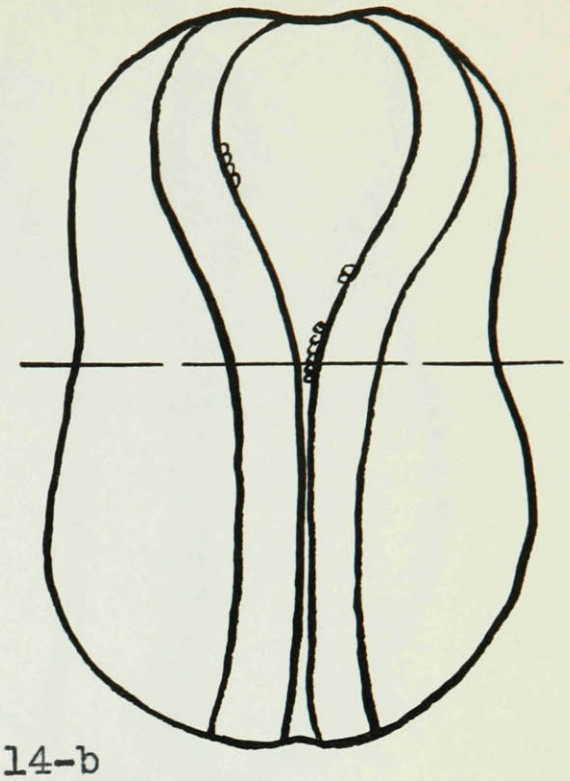
Fig. 13. Control. Same age as embryo shown in figure 12. (mag. app. 39x).

The same sequence of events, histologically, occurs in concentration 4.26. Here, however, the degeneration begins along the neural folds, or more specifically it appears to begin at the neural crest region of the neural folds (figure 14). It is at first fairly superficial (figure 15); later it involves notochord and adjacent mesoderm (figure 16); and finally, after complete destruction of the presumptive neural tissue, the degeneration spreads over the body ectoderm, causing consequent increase in gross size as has been noted before (figure 17).

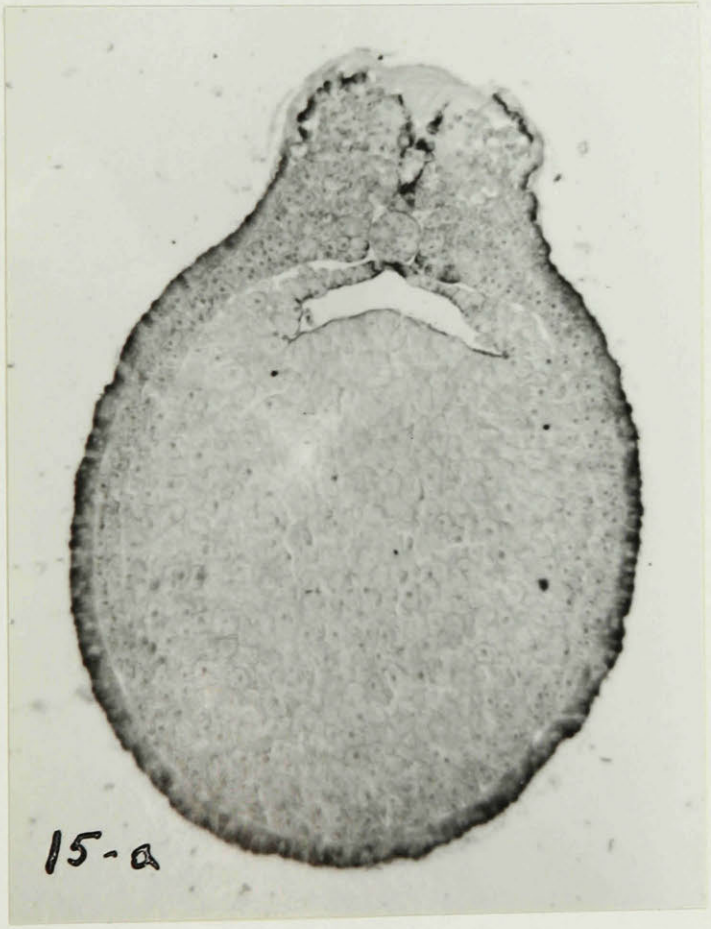
Figure 18 shows the approximate stage of development of the control animals at the time when the degeneration of the 4.26 animals becomes complete. It is at this same time that degeneration first appears in the 2.13 animals, in those cases where it does take place.

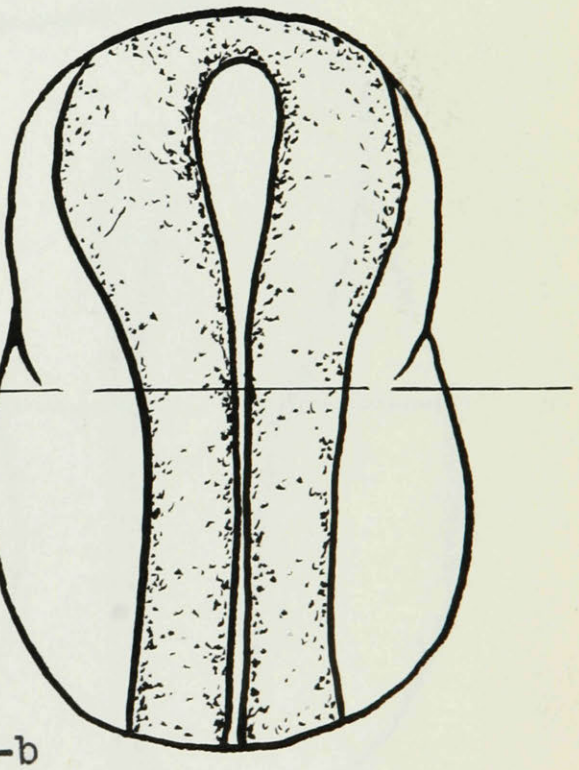
As may be seen from figure 19, degeneration of the 2.13 animals is restricted not only to the neural fold tissue, but, even more specifically, to the median surfaces of the permanently open neural folds; that is, it affects the thickened tissue which would have gone to form neural tube if closure had not been prevented.





14-b

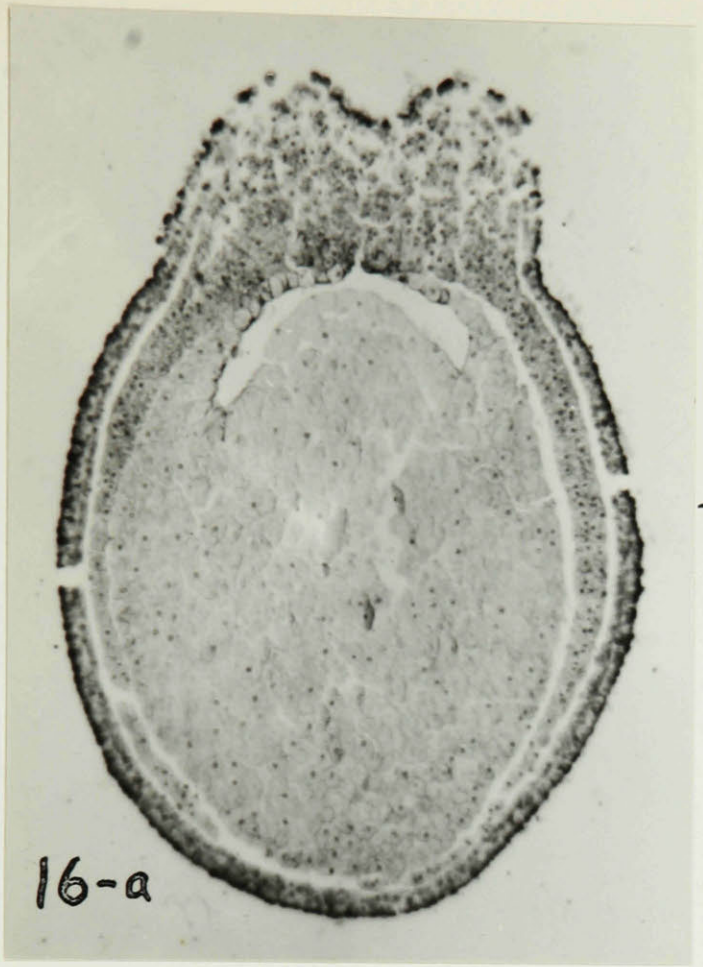


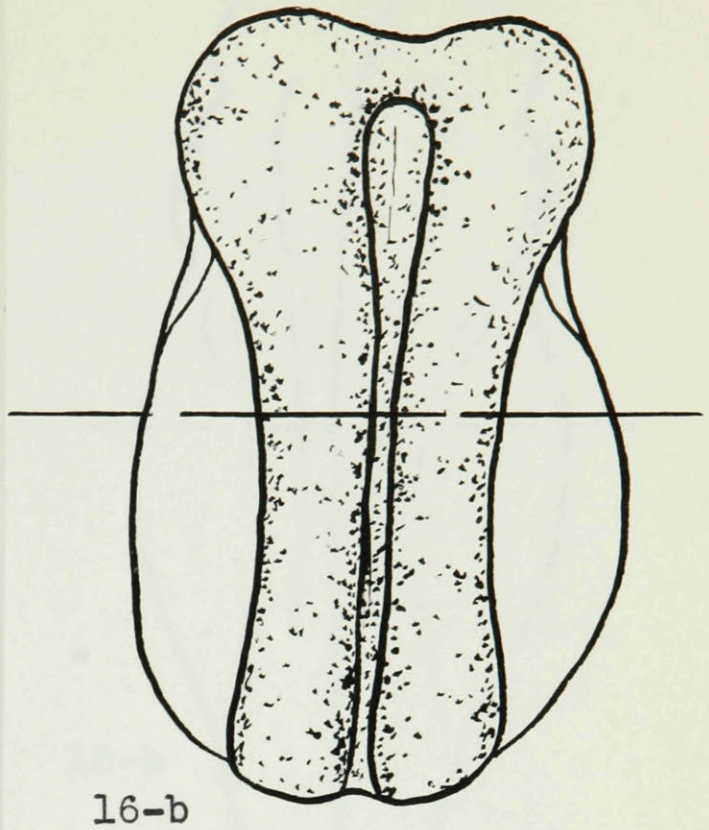


15-b

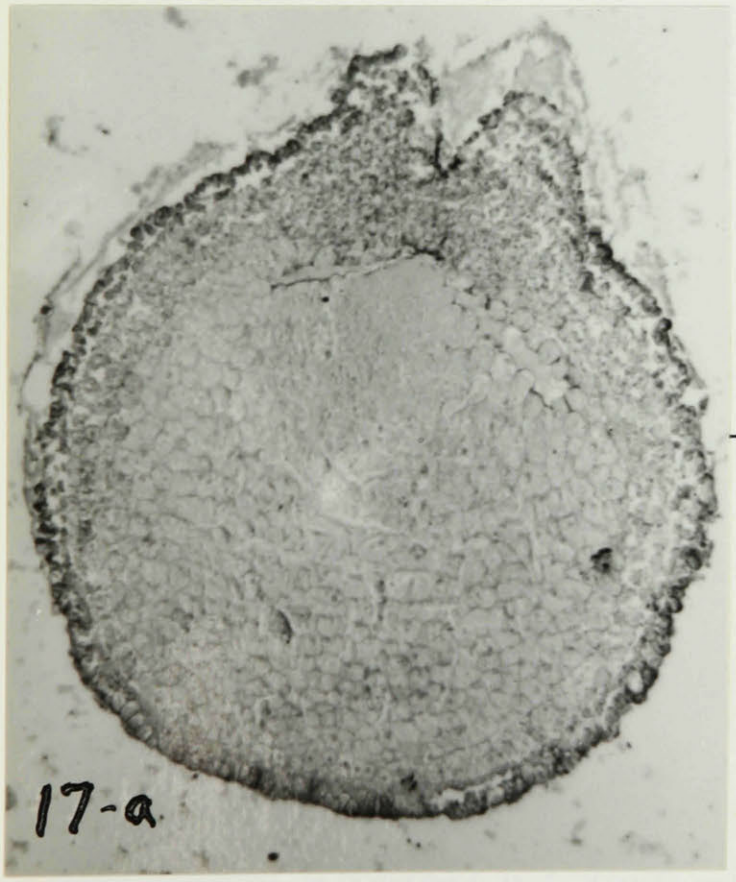
Fig. 14. (Conc. 4.26). Earliest degeneration. An isolated, swollen cell may be seen protruding beyond the normal margin of the neural fold (14-a).

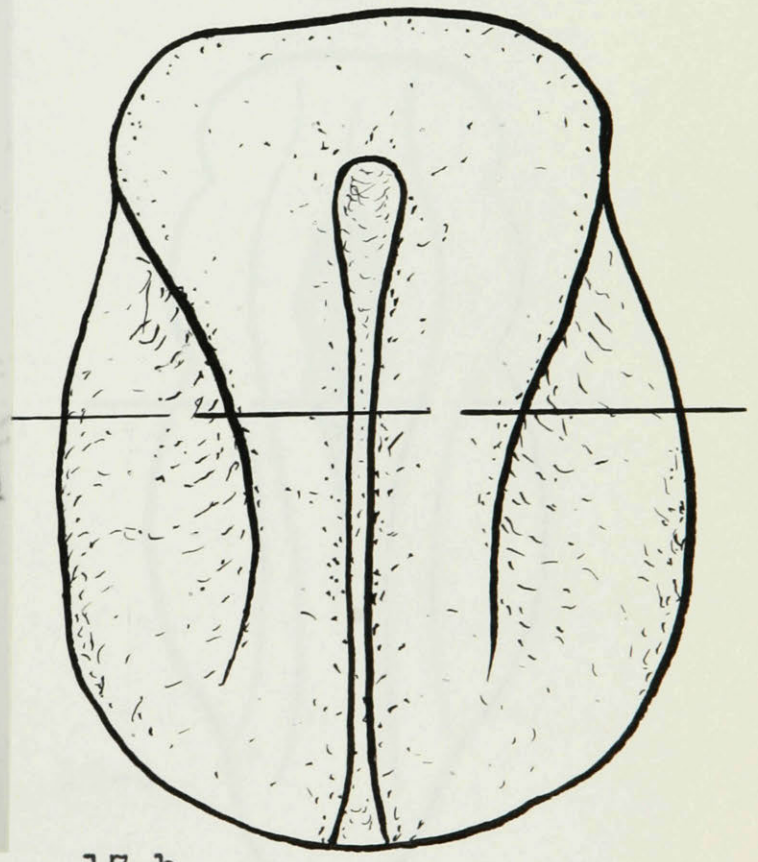
Fig. 15. (Conc. 4.26). Degeneration has spread along the neural folds. Some pigment recession, as well as rounding up and breaking away of cells may be seen. (Mag. app. 39x).





16-b

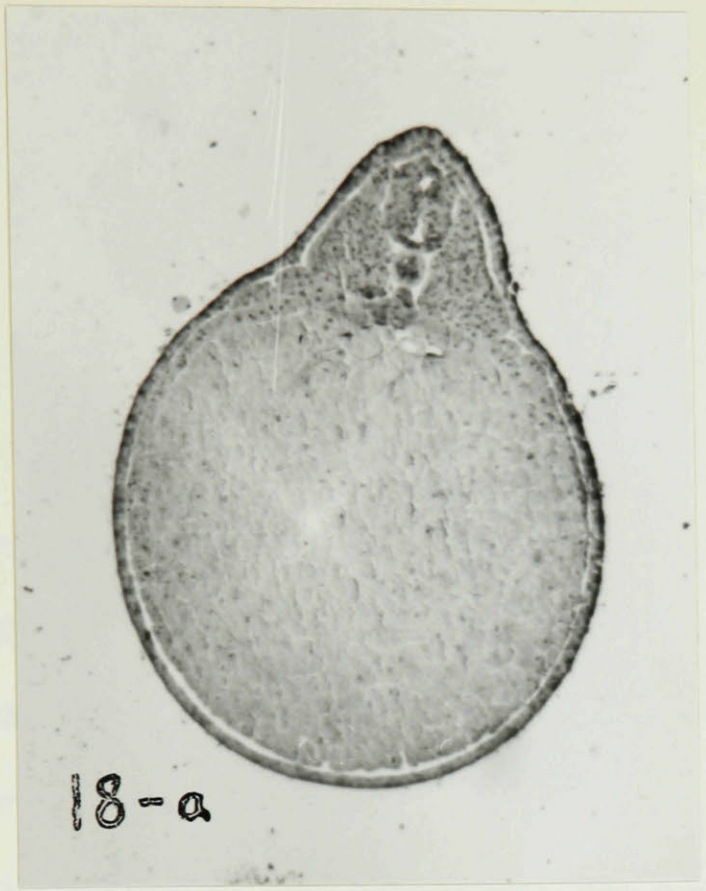


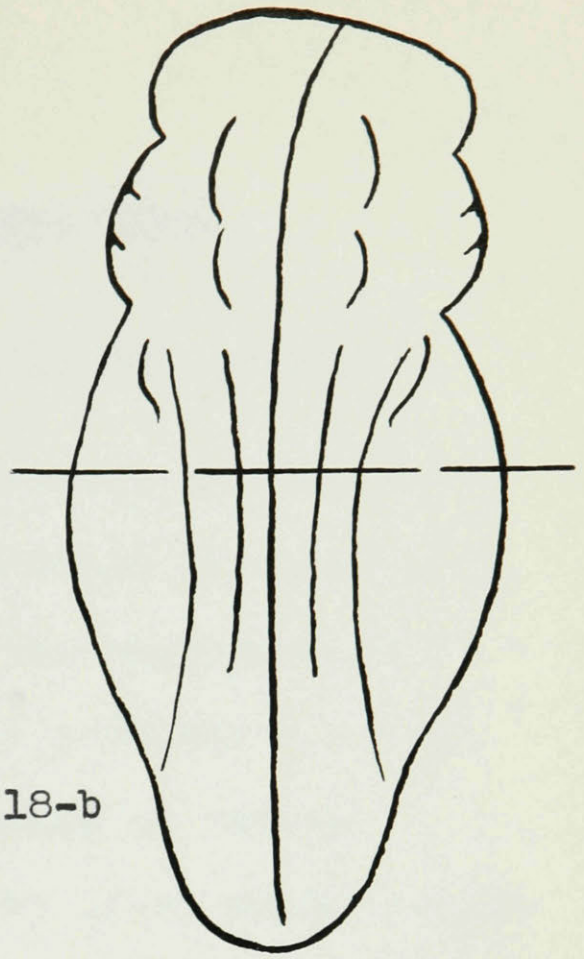


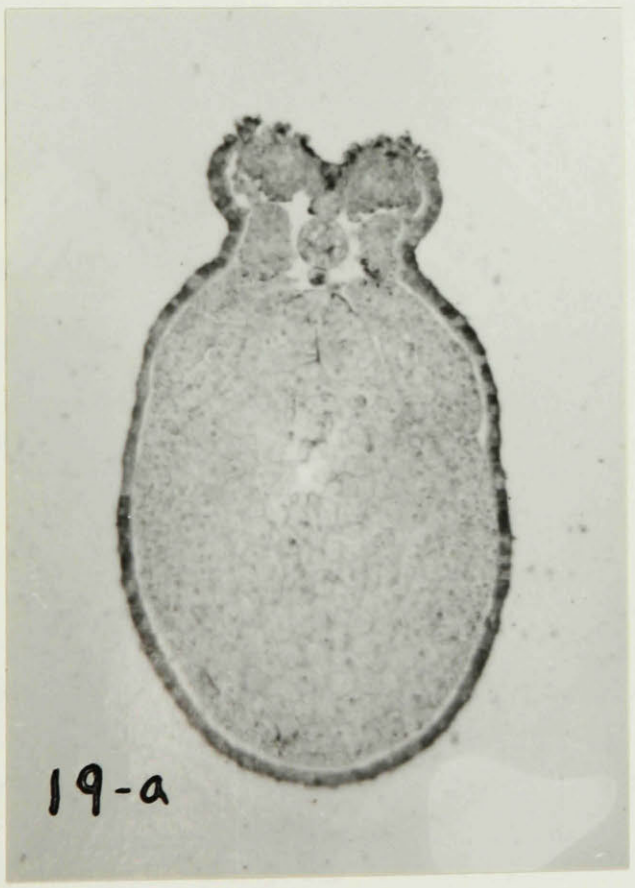
17-b

Fig. 16. (Conc. 4.26). Degeneration has spread more deeply to include the notochord and adjacent mesoderm. In surface view, however, it is still confined to the neural folds.

Fig. 17. (Conc. 4.26). Total degeneration. Body ectoderm becomes affected only after neural fold destruction is complete. Note increase in size of embryo, due in part, at least, to swelling of cells.
(Mag. app. 39 x).







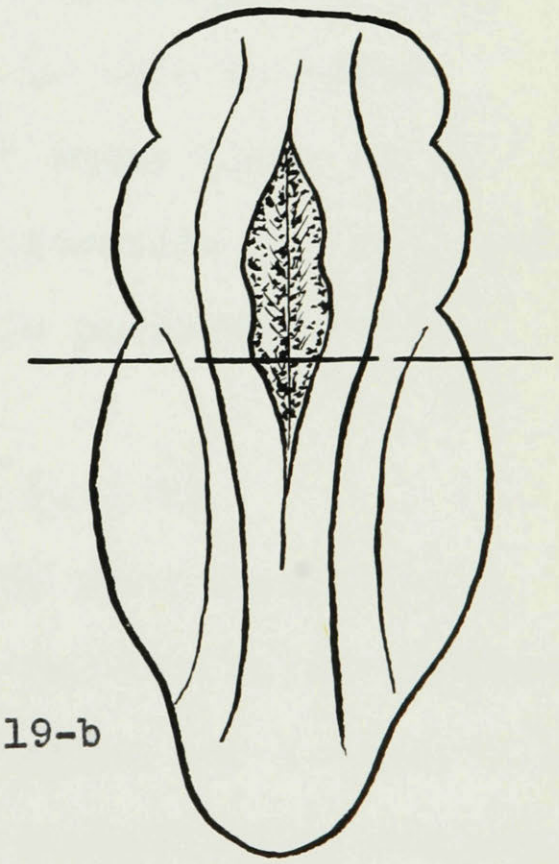


Fig. 18. Control; same age as embryos in fig. 17 and 19.

Fig. 19. (Conc. 2.13). Early degeneration, confined, at this stage, to the permanently open region of the neural groove.
(Mag. app. 39x).

II. SUBSIDIARY EXPERIMENTS

A. Analysis of time of action of the carcinogen

In the experiments just described, it will be remembered that the carcinogen produced no observable effect upon the eggs until after the beginning of gastrulation. It was thought that possibly a latent period was responsible for this delay of action. Accordingly, it occurred to us that if we could expose the eggs to the carcinogen for a considerable period of time before cleavage, we might be able to bring about an effect on cleavage itself which might offer opportunity to observe not only alteration of rate, but possibly also some upset of mitotic phenomena in the cleaving egg.

1) Injection of DBSA in vivo (Exp. 18).

With this hope we injected the carcinogen intraperitoneally into female frogs at periods which provided exposure of the eggs to the hydrocarbon for 4 days, 8 days, and 1 month before fertilization, respectively. Controls were frogs which were either uninjected or injected with an equivalent amount of Ringer's solution at corresponding periods.

4 day exposure: (1 mg. in 1 cc. Ringer's).

There was acceleration of cleavage and beginning of gastrulation relative to the two control groups. However since each group of eggs was from a different female it is doubtful whether this difference in rate was at all significant. Later development was completely normal and in all respects like that of the controls.

8 day exposure: (1.212 mg. in 1 cc. Ringer's).

There was no effect on rate of development, or on the total lengths of the animals at the end of the experiment. However, at certain points during the experiment there appeared to be a marked effect on physiological development. Thus, when all animals were at stage 24 (gills showing on left side only) the controls were arranged at the periphery of their dishes in characteristic fashion, and all swam very actively when the dishes were agitated. The experimental animals, on the other hand, were all lying at random positions at the bottom of the dish. Upon agitation, the majority showed no response while the few most active ones swam two or three times their length and then stopped. It is possible

that these differences may be due to the fact that the dibenzanthracene had a specific effect on the nervous system at some previous stage. However it is impossible to draw any conclusions without repitition of the experiment and histological analysis of nervous system differentiation.

1 month exposure: (0.727 mg. in 0.6 cc. Ringer's).

The results of this experiment were very striking although difficult to interpret. The eggs of this female were a mixture of large degenerated grey ones and smaller, superficially normal-appearing ones. Only the latter were used for the experiment.

Cleavage looked normal from the animal pole; the yolk material, however, showed few or no cleavage furrows and had streaks of pigment running into it giving it a marble-like appearance.

Beginning of gastrulation was somewhat delayed, and when it did occur it often took place at as many as five points at once. These multiple dorsal lips persisted for a time, after which the pigmented cells contracted and formed a small cap of folded ectoderm on top of the yolk mass, giving the eggs the appearance of exogastrulae. Subsequently the yolk became vacuolated and cytolyzed.

The ectodermic cap either fell into the cavity so formed, or was actually engulfed by the yolk material, and the egg died and disintegrated. (Figure 20 shows this sequence of events). In no case was there any suggestion of the type of degeneration observed in the previously described "basic" experiments.

2) Fertilization in DBSA

The next experiment (#22) aiming at analysis of the time of action of the carcinogen consisted of eggs from a single female which were divided into four groups and treated as follows:

(a) control - fertilized in, and subsequently kept in 1/10 Ringer's.

(b) DBSA #1 - fertilized in, and subsequently kept in concentration 10.0 DBSA.

(c) DBSA #2 - fertilized in 1/10 Ringer's, and transferred to 10.0 DBSA 18 minutes post fertilization (thus eliminating the possibility of the carcinogen acting directly on the sperm).

(d) 1/10 Ringer's - fertilized in 10.0 DBSA, and transferred to 1/10 Ringer's 2 hours post fertilization.

Results: - The control and 1/10 Ringer animals developed exactly alike. Thus no harmful effect whatsoever

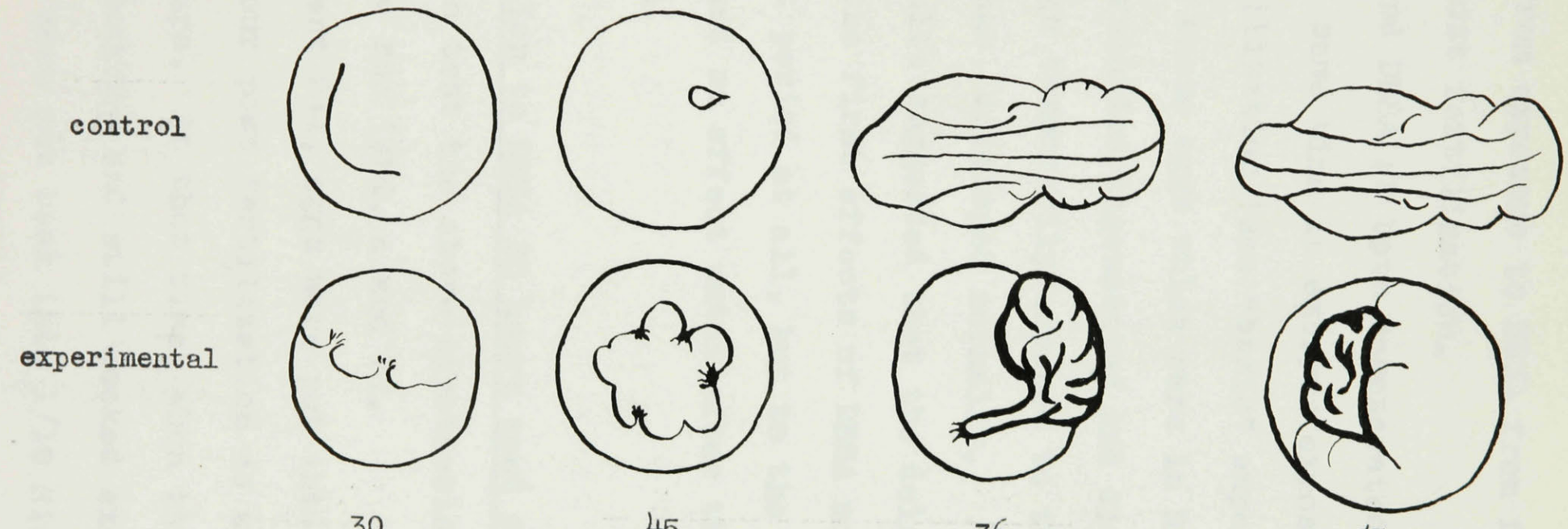


Figure 20. Effect of exposure of eggs in vivo to DBSA for one month prior to fertilization.
(Camera lucida drawings. 13.2 x).

had occurred from exposure to DBSA from fertilization to two hours post fertilization.

DBSA #1 and DBSA #2 both degenerated in the same way and at the same time as embryos exposed from one hour post fertilization (see "basic" experiments). In short, only those eggs which were in DBSA during and after gastrulation degenerated and died, while others, although temporarily exposed to the carcinogen before that stage, developed normally.

These results suggested that the delay in the appearance of the first effects of DBSA might not be due to a latent period at all, but to the fact that the DBSA actually had no effect until after the initiation of gastrulation.

3) Subjection to DBSA 25 hours post fertilization

In order to test the above hypothesis two more experiments were run (#24, a and b).

In experiment 24a, eggs were put into concentration 10.0 DBSA one hour post fertilization as usual, and left there for 25 hours. At that time, when they had small crescentic blastopores and still looked exactly like the controls, they were put back into 1/10 Ringer (with several rinses). Subsequent development was exactly like

that of the controls, indicating that the carcinogen actually had not exerted any marked or irrevocable effect up to and through the beginning of gastrulation.

Experiment 24b was the converse of 24a. Eggs were reared in 1/10 Ringer's and transferred to 10.0 DBSA when they reached the small crescentic blastopore stage. Subsequently these eggs showed typical dorsal lip degeneration, which spread over the surface and caused the death of the embryos.

The results of these two experiments indicate that a long latent period is not involved, and that the carcinogen actually acts upon the egg after gastrulation has begun. (It is well known that there is, at the time of gastrulation, a sudden increase of metabolic processes. Also it is at approximately this stage that there is a shift from anaerobic to aerobic glycolysis (Brachet '35-b). It would be interesting to do further experiments to determine whether either of these changes has any direct correlation with the development of sensitivity to the carcinogen.).

B. Effect of the carcinogen on post neurula embryos

In experiment 11 embryos were placed in the carcinogen just at the time of neural fold fusion (approximately

stage 16). Concentrations 0.213, 2.13, and 4.26 were used, and each animal was numbered and kept in a separate bottle in the thermostat for the purpose of measuring growth rates of individual animals. Each bottle contained 10 cc. of solution which formed a layer about 1.4 cm. deep. The solutions were aerated twice each day, and daily growth measurements were made of tail, trunk, gills and total length by means of an "embryometer" (appendix II).

The 4.26 animals were much retarded and died shortly after being put in the solutions. The animals in concentration 2.13 showed definite progressive retardation, and four days after subjection to the carcinogen the tails of these animals began to show a "pebbly" surface and degeneration.

Figure 21 shows the growth curves from this experiment and figure 22 shows a cross-section of a tail region of an animal which had been in concentration 2.13 for four days. Ventrally the cells are seen to be degenerating and sluoghing off. Laterally there is a thickening and folding of the epithelium which is responsible for the "pebbly" appearance of the surface in the living animal. This type of change is frequently observed in areas which subsequently degenerate. Thus it appears to be a type of pre-degenerative hyperplasia.

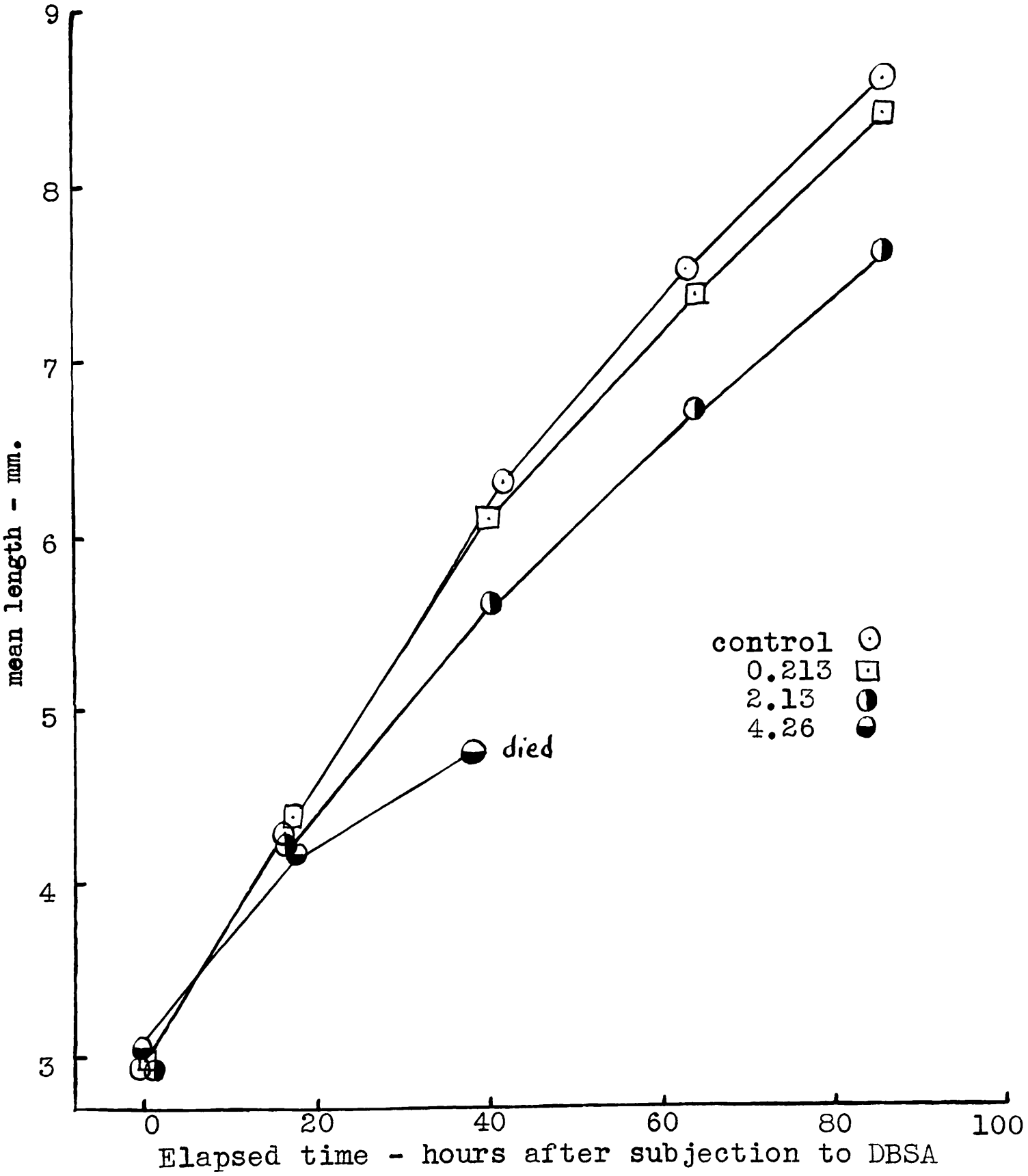


Fig. 21. Growth curves of animals placed in DBSA solutions at time of neural fold fusion.

Fig. 22

Fig. 22. Cross-section of the tail of an animal which was in DBSA solution (concentration 2.13) for four days, having been placed in the solution at the time of neural fold fusion.

Ventrally, degeneration may be seen. Laterally there is pre-degenerative hyperplasia of the ectoderm which, in the living animal, gives a "pebbly" appearance to the surface.

(mag. app. 108 x).

C. Effect of DBSA on tadpoles

The animals used in this experiment (#12) were Rana sylvatica, which were collected in April from a swampy region west of Montreal. They were at tail-bud stage when collected and were put in solutions eleven days later when they were completely formed tadpoles (average total length, 10mm.). As in experiment 11 the animals were kept in individual dishes. In this case finger-bowls were used with 25cc. of solution in each. The controls were kept in 1/10 Ringer's and the experimental animals in 4.0mg./100cc. DBSA. The animals were fed boiled lettuce and the solutions were changed after seven days.

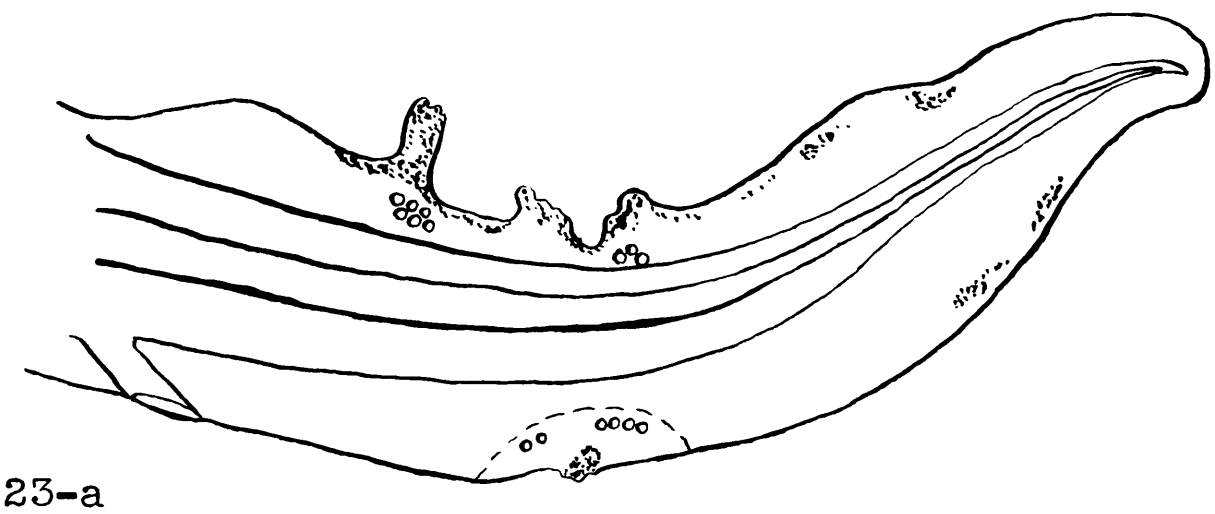
All animals were weighed and measured at the beginning of the experiment and three times during the following six days. From that time on, however, it became increasingly difficult to make accurate measurements because of distortion of the shapes of the tails of the experimental animals. Therefore camera lucida drawings were made instead. The experiment was terminated after twelve days at which time measurements were made of all the controls (20) and of the 5 (out of 20) surviving experimental animals.

The effects of the DBSA solution observed during the course of the experiment were: -

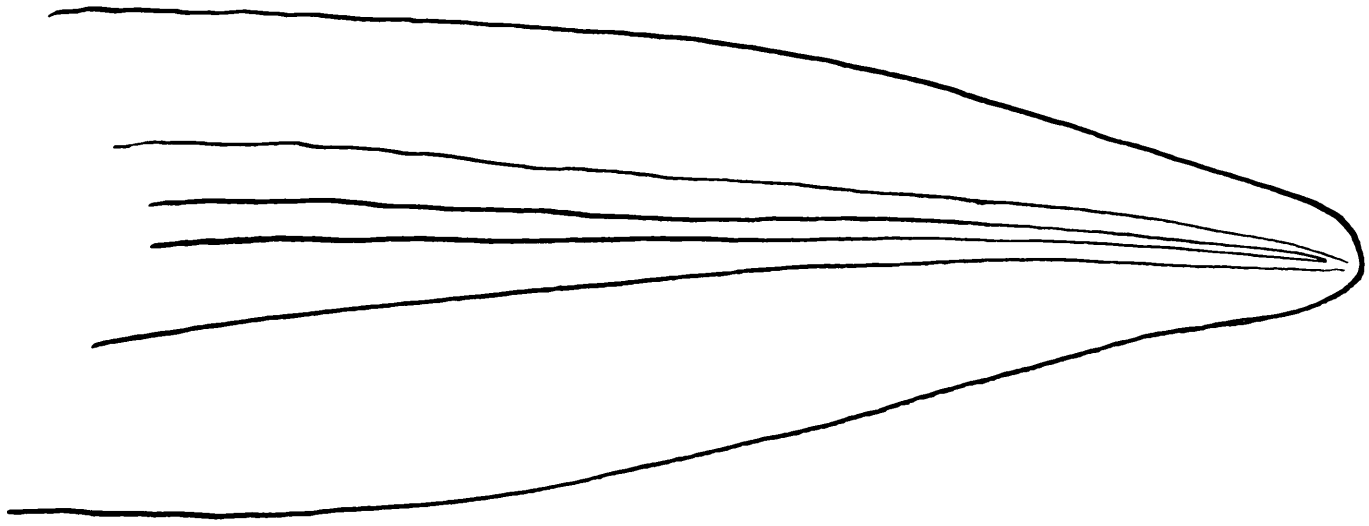
1) Retardation of growth. The experimental animals were not only smaller than the controls, but they became very emaciated although fed the same amount as the controls.
2) Surface injury. The DBSA appeared to act as an irritant and caused extensive necrosis of tail fin and body ectoderm, as well as hemorrhages and blisters in the tail. Injury to underlying muscle tissue of the tail was probably responsible for the distortion of its shape.
3) Lethal effect, presumably due to hemorrhage and tissue injury.

Figure 23 shows camera lucida drawings of the tails of typical experimental and control animals.

Subsequent sectioning showed certain areas, unobserved in the living animals, which appeared to be undergoing active proliferation. Such areas (shown in section in figures 24, 25 and 26) are apparently unrelated to the necrotic regions. Although the proliferating tissue appears to be continuous with the ectoderm, it is certainly not typically ectodermal in character; nor does it resemble the usual pre-degenerative folding of ectoderm seen in



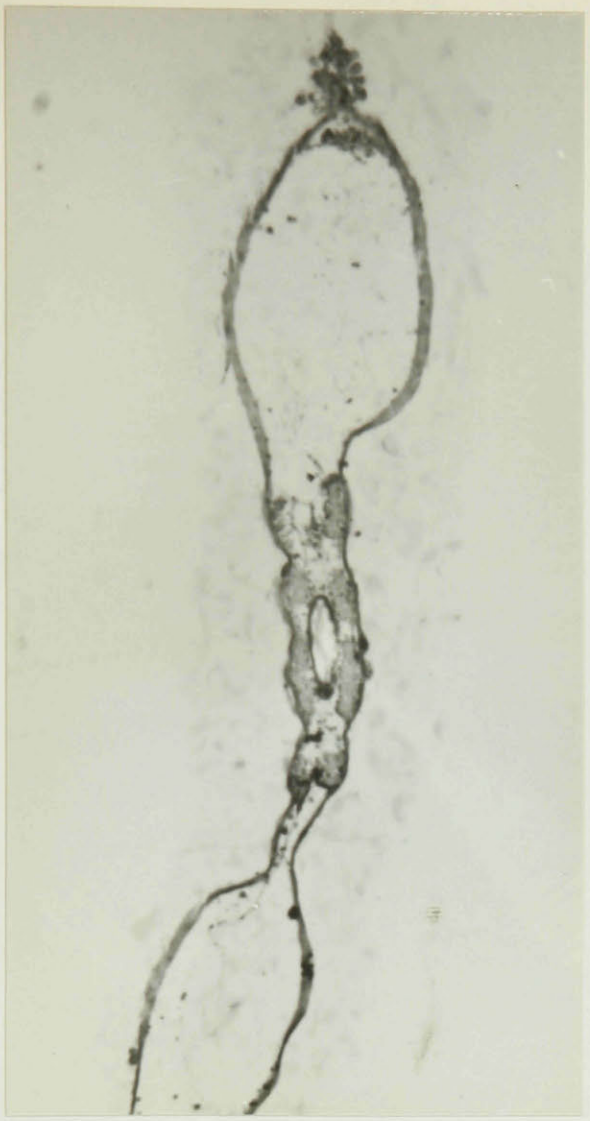
23-a



23-b

Fig. 23-a. Camera lucida drawing of the tail of a tadpole 8 days after being placed in a 4mg./100cc. solution of DBSA. Stippling indicates necrotic areas which are in some cases sloughing off. Small circles indicate areas of hemorrhage; dotted line outlines a blister. (Mag. 13.2 x).

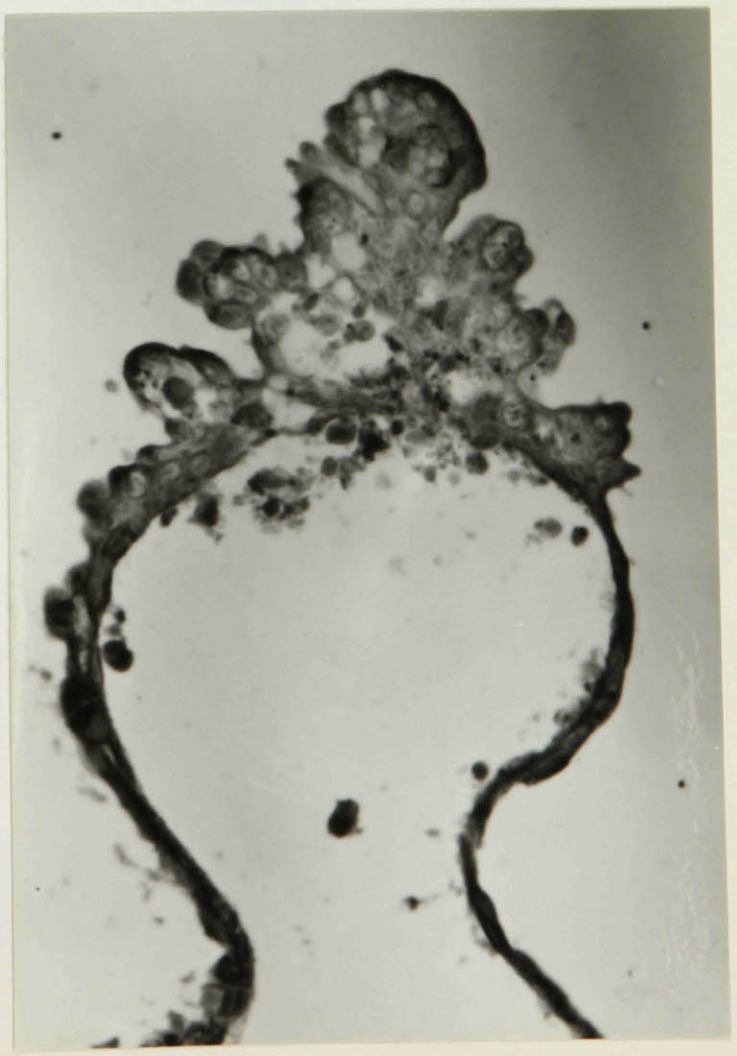
Fig. 23-b. Camera lucida drawing of the tail of a control tadpole of the same age as the animal shown in 23-a. (Mag. 13.2 x).



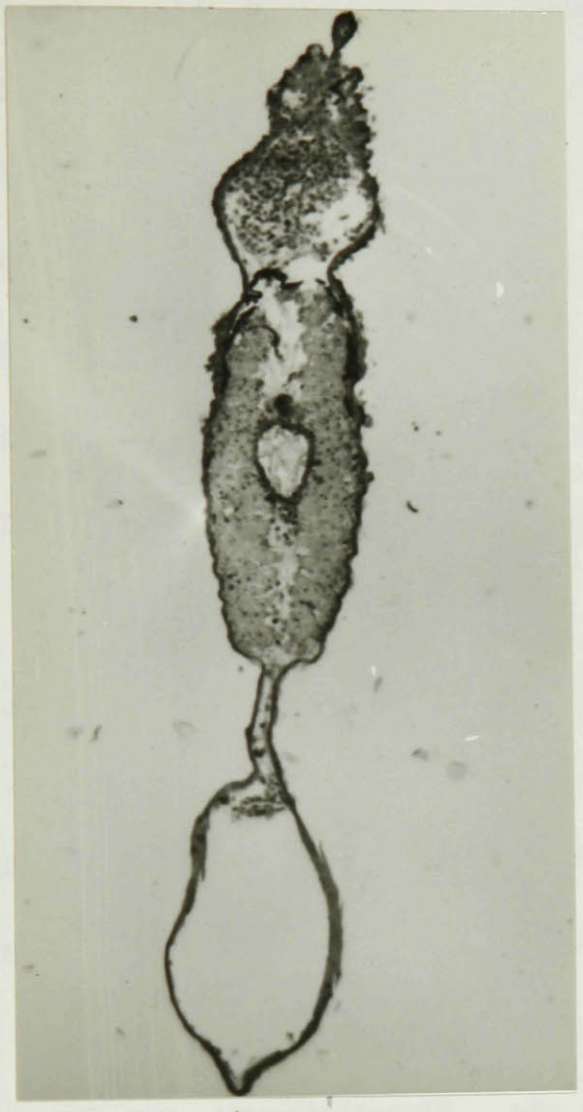
24-a

24-b

25-a



25-b



26-a

26-b

Figures 24-a, 25-a, and 26-a show cross-sections of the tail of a tadpole which had been in a 4mg./100cc. solution of DBSA for 9 days. Large empty spaces within the tail fin indicate "blisters". Proliferating areas in each case may be seen at the edge of the fin. (Mag. 67x).

Figures 24-b, 25-b, and 26-b show the corresponding proliferating areas at higher magnification (270 x). There is no sign of degeneration but rather an active papilloma-like outgrowth.

figure 22. It appears rather like a papillomatous type of growth, and it is perhaps noteworthy that the animal, of whose tail these sections were made, had been exposed to the carcinogen for only nine days. So far only this one tail, from experimental animal #7, has been sectioned, but the change seen here would appear to warrant further study.

D. Effect of use on the activity of DBSA

It was thought pertinent to determine whether or not the solutions lost any of their potency during the course of an experiment. Aside from affording information as to the necessity of renewing solutions during the course of an experiment, we thought that such an experiment might throw some light on the question of the mechanism by which the carcinogen acts; i.e. is it actually "used up" (which might imply that it is metabolized or somehow converted by the animal), or does the solution retain its original potency ? (which might imply that it acts more in the nature of a catalyst, being unchanged and undiminished at the end of the reaction).

Reports of various workers tend to support the first possibility: Heiger ('36) found that tumors appeared in a certain group of DB-treated animals 20 weeks after the

last skin painting, which was 16 weeks after the carcinogen ceased to be detectable by fluorescence methods (sensitivity, 1 millionth to 1 ten millionth gram).

Berenblum and Kendall ('36) injected known amounts of DB into mice and subsequently made measurements of a) amount at site, b) amount excreted, and c)amount in the whole body. The values of these three measurements did not equal that of the original injections. Therefore the authors concluded that the DB must be actually altered in the body, i.e. metabolized into a form no longer possessing the characteristic fluorescent bands.

Additional evidence in support of such a conclusion is presented by Dobriner et al ('39) who studied the fate of DB injected into rabbits, rats and mice. In the excreta of all animals injected they found certain substances of a phenolic nature, considered by them to be a conversion product of DB. (Incidently, they found also that the substance from injected rabbits was non-carcinogenic and gave absorption bands different from those of substances from rats and mice, suggesting that different species metabolize DB differently).

In our experiment,(#15, a and b), in order to eliminate a) the effect of different ages of the solutions to be

compared, and b) possible variations among different batches of solutions, it was decided to make up a double amount to begin with. Half of each solution (25cc. of each concentration) was set aside on a shelf at room temperature, the other half being used in experiment 16 (one of the "basic" experiments). During experiment 16, animals in the stronger concentrations were removed at the very first sign of degeneration, to prevent possible contamination of the solutions by toxic substances. At the end of experiment 16, solutions from the control and concentrations 4.26 and 10.0 dishes were filtered and measured. The effect of these used solutions was then compared with that of the corresponding unused solutions of the same batch and age. Concentrations 4.26 and 10.0 were chosen for comparison because they had been shown to exert a specific effect beginning at a definite developmental stage.

Results: - In brief, development in all cases was exactly alike in the used and the corresponding unused solutions. In concentrations 4.26 and 10.0, degeneration began at the same time in corresponding dishes and progressed at approximately the same rate.

From these results the following conclusions may be drawn: either a)the solution is not "used up", or b) the

solution is "used up" in bringing about its effect, but under the conditions of the experiment (concentrations, amount of fluid and number of eggs per dish, etc.), there is still sufficient quantity of the active agent or agents left over, after affecting one group of eggs, that the carcinogen is capable of acting again, fully, on a second group.

In either case, the experiment demonstrates that the solutions retain their full potency even after eggs have been exposed to them for a period of a week. Thus it may be assumed that renewal of solutions, except for experiments of significantly longer duration, is unnecessary.

E. Effect of age on the activity of DBSA

The purpose of these experiments (#17, a and b), was to determine whether there was any loss of potency when the solutions were kept 1) at room temperature (20-23° C.) and 2) in the icebox (4° C.).

In experiment 17-a the following unused solutions were used:

a) control
b) 4.26; 8 months old; kept at room temp.
c) 4.26; 8 days old; kept in icebox.
d) 10.0; 7 months old; kept at room temp.
e) 10.0; 18 days old; kept at room temp.
f) 10.0; 8 days old; kept in icebox.

It was assumed at the beginning of the experiment that the 8-day-old icebox solutions were equivalent to newly-made ones, and would therefore serve as a basis of comparison for evaluating the strength of the older solutions. However this did not prove to be the case.

4.26 (8 mOnths) was definitely less effective than 4.26 (8 days-icebox). However in both groups the embryos completed neural fold fusion, which was never the case in the 4,26 solutions of the "basic" experiments.

Similarly, in concentration 10.0 (7 months) there was some closure of blastopores, indicating definite weakening of the solution. In 10.0 (8 days-icebox), although the yolk plugs persisted, there was definite neural fold formation before the embryos died. The 18-day-old-room-temperature 10.0 was the only one of the three which acted as in the "basic" experiments, i.e. prevented both closure of blastopore and formation of neural folds.

It was understandable that 7 and 8 months ageing might cause some decrease in potency, but the decrease due to 8 days in the icebox was surprising. Experiment 17-b was therefore run to confirm this latter point.

For 17-b,150 cc. of concentration 10.0 were made up. 50cc. were used while the solution was fresh

(for experiment 19, a and c). 50cc. were put in the icebox for 7 weeks, and the remaining 50cc. were kept at room temperature for the same length of time. At the end of this period, the solution kept in the icebox showed a considerable amount of flocculent, bluish precipitate.

The results of the experiment were as follows: -

a) 50cc. fresh (#19, a and c)
no closure of blastopore;
no suggestion of neural folds;
death at stage 11-12.

b) 50cc. 7 weeks-room temperature (#17-b)
no closure of blastopore;
suggestion of neural folds and neural groove in some embryos;
died at approximately stage 12.

c) 50cc. 7 weeks-icebox (#17-b)
completely normal development through neural fold formation; then beginning of degeneration of neural folds;
death at stage 14-15.

The variations between a) and b) may be readily explained as due to variation in hardiness of different batches of eggs. Thus the ageing of solutions at room temperature, up to seven weeks at least, has no appreciable effect.

Cold, however, apparently lowers the solubility threshhold, and consequent precipitation of some of the DBSA results in marked decrease in the potency of the

solution. In experiment 17-a the 4.26 (icebox) solution acted approximately like a newly-made 2.13 concentration. (Although precipitation was not grossly noticeable, it had evidently occurred to some extent). In experiment 17-b the 10.0 concentration (icebox) allowed development of well-formed neural folds, thus acting roughly like a newly-made 4.26 solution. In other words, precipitation due to cold had reduced the potency of the solutions by approximately 50 %.

F. Effect of light on the activity of DBSA

Various workers have observed that the presence or absence of light affects the activity of carcinogens.

M.R. Lewis ('35), in observing the effects of a suspension of DB on cultures of chick embryo tissue, found that cells appeared normal when the cultures were studied under dull light, but that the cells became changed and mitoses inhibited within 2-10 minutes after exposure to bright light.

Hollaender et al ('39) found that methylcholanthrene in the dark stimulated the growth of yeast, whereas methylcholanthrene in light (near ultra-violet radiations) had a toxic effect on yeast (greater than that of near ultra-violet radiations alone).

Mottram and Doniach ('37) and Doniach ('39) observed the photodynamic action of certain carcinogens on cultures of Paramecia. They found that Paramecia without the carcinogen (benzpyrene) showed no effect after some hours under a lamp. Paramecia exposed to benzpyrene in the dark showed no lethal effect after weeks of contact. However animals with benzpyrene died within a few minutes when exposed to light.

Velluz ('38) and Cook, Martin and Roe ('39), testing the theory that carcinogens may act by primary conversion to water-soluble oxidation products, found no evidence that such a type of light sensitivity was of significance in connection with the carcinogenic activity of the hydrocarbons tested.

In view of this contradictory evidence on the subject it seemed worthwhile to run an experiment with our material to observe 1) whether development would proceed, in concentration 10.0 for example, without retard or degeneration when the embryos were exposed to solutions in the dark; and 2) whether photo-oxidation products formed in our solutions were responsible, to any large degree, for the effects observed.

The experiment (#23, a and b) was run as follows: - 200 cc. of concentration 10.0 were made up, and 50 cc.

of this were put into each of four bottles. Two of these were kept on a shelf where they were exposed to daylight, direct sunlight, and artificial light at night. The other two bottles were immediately covered with several thicknesses of black paper (to prevent possible formation of photo-oxides). The solutions were kept in bottles for twelve days, after which they were transferred to dishes two of which were kept in total darkness inside containers, - a coffee can and a photographic developing tank. The experiment thus tested the effect of four different conditions: -

a) solution kept in light and used in light
b) " " " " " " " dark
c) " " " dark " " " light
d) " " " " " " " dark

In brief, the results indicated that neither keeping nor using the carcinogen in the dark in any way lessened or altered its activity.

III CONTROLS

A. Lowered surface tension; Aerosol O.T.

It was observed, in making up the DBSA solutions, that the higher concentrations showed frothing which was taken as evidence of lowered surface tension.

Several references to the lowering of surface tension and its possible significance were found in going through the literature: -

Bauer ('23) attempted to demonstrate that all carcinogenic agents lower surface tension, and that this lowering is the general cause of the "cancerisation" of a cell.

Reiss ('24), who reared sea urchin eggs in an emulsion of tar and sea-water, observed that the common feature of all the abnormalities so produced was a lack of cohesion of the blastomeres, which in turn he believed to be due to a lowering of the surface tension of the medium.

Katzenstein and Knake ('31), working with tissue cultures, observed that the addition of cholesterol or other surface tension lowering substances to the medium reversed the normal relationship of epithelial and connective tissue growth, stimulating the former and

causing restraint and disturbance of the latter.

Traube ('31), in commenting on the above work, pointed out its value and asserted that substances which lower the surface tension of body fluids and thus affect cell permeability, are a factor in the etiology of cancer.

Kopaczewski ('35), also working along these lines, found that carcinogens lower the surface tension of serum. He also observed that sarcoma serum appeared to have a lower surface tension than normal serum, and suggested that carcinogens might actually act by lowering surface tension.

With these facts in view, it was decided to determine the degree of surface tension lowering in our solutions, and to run a control with some non-toxic substance which would lower the surface tension to the same degree. (experiment #13).

The substance chosen was the dioctyl ester of sodium sulfosuccinate, known commercially as Aerosol O.T.. Lorenz et al ('40), using Aerosol in the preparation of dispersions of carcinogens and hormones, tested its toxicity and found that adult mice would tolerate doses of

0.2cc. of a 5% solution subcutaneously;
0.5cc. of a 0.2% solution intra-peritoneally;
0.25cc. of a 0.5% solution intra-venously;
800.0cc. of a 0.1% solution orally (4 cc. daily).

Surface tension measurements for our experiment were made by means of a du Noüy tensimeter. The usual dilutions of DBSA were prepared as well as a series of Aerosol solutions, and surface tension curves were determined for both. The solutions used in the experiment with their surface tension values (dynes per centimeter) were:

control #1 control #2	average	73.38 ± 0.3
DBSA 10mg./100cc. "		62.04 ± 0.2
Aerosol 0.2mg/100cc."		59.39 ± 0.5

pH's were also checked and found to be 7.0-7.2 in all cases.

Greyish exovates appeared in the DBSA embryos and typical degeneration began at approximately 40 hours after fertilization. The embryos in Aerosol and 1/10 Ringer's never showed either exovates or degeneration, their development being completely normal and identical throughout.

At the end of the experiment, when control and Aerosol animals had reached stage 25 (fully formed tadpoles) the surface tension of the three solutions was again measured, in case any changes might have taken place. All values

were slightly higher, due probably to temperature difference, but they had remained constant relative to each other:

controls	average	73.99 ± 0.3
DBSA 10mg/100cc.	"	64.42 ± 0.6
Aerosol 0.2mg/100cc.	"	61.31 ± 1.2

Thus, in summary, it was concluded that the capacity of the DBSA to lower surface tension was not responsible for the effects produced in these experiments.

B. Sodium succinate control

Various workers (Boyland '33; Pourbaix '33-b; Deotto '36) have reported that carcinogens affect the respiratory mechanism of cells. Pourbaix ('33-a) suggested specifically that interferrence with cell respiration might be the mechanism by which carcinogenic agents act.

Boyland and Boyland ('36) observed that respiration (of tumors, as well as of normal tissues - muscle, kidney, liver) is affected by the sodium salts of some dibasic acids. Sodium succinate was found to increase respiration.

In order to eliminate the possibility that our effects might be due to the sodium succinate part of the Na-salt of DBSA used in these experiments, an experiment (#14)

was run using a 10mg/100cc. concentration of DBSA and an equimolar (7.02mg/100cc.) solution of sodium succinate.

The animals in the carcinogen showed typical early degeneration, while those in sodium succinate and 1/10 Ringer's developed perfectly normally and alike in all respects. Thus it may be assumed that the effects observed as a result of treatment with Na-1,2,5,6-dibenzanthracene-9,10-endo-α,β-succinate are not in any way due to the Na-succinate part of the molecule.

C. Related non-carcinogen: Anthracene

The purpose of this experiment (#21) was to determine whether the effects we had observed might be considered as due specifically to the carcinogenic properties of the DBSA. In other words, would a closely related, but non-carcinogenic hydrocarbon produce the same effects ?

The choice of substances which might be used as a control was limited to those 1) which were related to DB, 2) which were known to be definitely non-carcinogenic, and 3) of which the endo-succinate derivative could be formed. From a considerable list of substances which satisfied the first two conditions, only six satisfied the third (see appendix III). Of these six, the only one obtainable

was anthracene. The derivative, anthracene-9,10-endo-α,β-succinic acid, was made and purified by Donald Robinson,(Department of Chemistry, McGill), according to the method of Bachmann and Kloetzel ('38), and had a melting point of 152-155° C. (corr.). The trans form was not made because the sodium salt of the more usual cis form was known to be readily soluble (Warren '39).

The experimental set-up consisted of three dishes:

a) control

b) 10mg/100cc. DBSA

c) equimolar (7.46mg/100cc.) anthracene-SA

The results, in brief, were that the animals in DBSA showed the usual retard and degeneration, while those in anthracene-SA developed exactly like the controls throughout. Thus the effects produced by DBSA appear to be particularly associated with that (carcinogenic) compound, and not with the related (non-carcinogenic) compound, anthracene. This fact, together with the two previous control experiments, supports the hypothesis that the carcinogenicity of the DBSA is responsible for the effects observed.

D. Anthracene - Aerosol

It was observed, while making up the solutions for the experiment just reported, that the anthracene derivative showed none of the frothing observed in the DBSA. A measurement of surface tension values at the end of the experiment gave the following results:

control	70.86 ± 1.0
anthracene-SA	71.06 ± 1.06
DBSA	62.83 ± 1.3

confirming that the anthracene did not cause any lowering of surface tension.

It then occurred to us that although anthracene alone had no harmful effect (exp. #21), and lowered surface tension alone had no effect (exp. #13), it might be that lowering of surface tension works in combination with some other factor. Specifically, it might be that DBSA acts as it does because (a) it lowers surface tension, and (b) at that lowered surface tension some component of the DB molecule is able to affect the egg.

Following this line of thought, it seemed possible that anthracene might contain the significant component, but that it was unable to be effective simply because of the fact that the surface tension was not lowered.

In order to test this entirely theoretical hypothesis

a solution of anthracene-SA was made up, diluted with 0.2mg/100cc. Aerosol, such that it was equimolar with 10mg/100cc. DBSA and at the same time had a surface tension value equal to (actually slightly below) that of the DBSA.

Surface tension measurements of the three solutions used for the experiment (#25) showed the following values (dynes per centimeter):

a) control	72.63 ± 0.2
b) 10mg/100cc. DBSA	62.68 ± 0.7
c) anthracene-Aerosol	59.86 ± 1.3

Result of the experiment: the development of the animals raised in anthracene-Aerosol was exactly like that of the controls in all respects.

E. Metabolic poisons

Boyland ('33) and others (mentioned in connection with the Na-succinate control experiment) have suggested that carcinogens may act by interferring with cell respiration. Certain other workers have observed that carcinogens may interfere with glycolysis as well.

Boyland and Boyland ('34), using tissue culture methods, observed that oxidized DB inhibited oxidation and glycolysis in both normal tissues and tumor tissue. (Inhibition, with one exception, occurred more slowly in the tumor tissue,

but otherwise the effects were the same).

Boström ('35) showed that, after a previous injection of dibenzanthracene, liver slices (of mice) show disturbances in oxidation and glycolysis when exposed to the carcinogen.

Pourbaix ('37), studying the effect of the water-soluble carcinogen, Styryl 430, on yeast, observed that not only was respiration reduced by certain concentrations, but also aerobic and anaerobic metabolism were inhibited by a concentration of 1:10,000. Pourbaix ('38) reviews her work from 1932 onward on the effect of carcinogenic compounds (including DB) on the carbohydrate metabolism of mammalian tissues and of yeast.

Neumann ('38) found that tissue metabolism was altered under the influence of 1,2-Benzpyrene.

Wright and Anderson ('38), studying the effect of dibenzanthracene on the fungus, Fusarium lini, found that certain concentrations caused a definite increase in the utilization of glucose in the medium.

Graffi ('39), observing the penetration of carcinogens into cells by means of a fluorescence microscope, found that the mitochondria appear to have a selective affinity for the carcinogens used. As a result of these and other findings, he suggested that "Carcinogens act by deranging

cell metabolism through their effects on the mitochondria rather than by attacking the chromosomes".

Further examples could be cited, but these serve to demonstrate that in many cases carcinogens have been observed to have a definite effect upon metabolic mechanisms.

In view of this, it seemed pertinent to compare the effects of DBSA with those of certain metabolic poisons of which something is known of the mode of action, and of their effect on frog embryos.

Brachet ('35-a; '35-b), and Brachet and Needham ('35) have studied the metabolic processes of the developing frog egg (R. fusca). Bellamy ('19; '22), Brachet ('34), and Needham and Needham ('40) have reported on the effects of certain metabolic poisons on early frog and, in the last case, sea urchin development. The substances and the concentrations which were used for our purposes (exp. #20) were chosen on the basis of the work of these authors.

a) KCN; inhibits utilization of molecular oxygen by poisoning oxydases, particularly cytochrome oxydase. Thus it affects some parts of aerobic metabolism.

Previous observations (KCN, M/10,000): Brachet ('34) - blastopore closes but development is much retarded. Bellamy ('22) - eggs subjected at two cell stage show extreme retardation and die with large blastopores (- "equatorial gastrulae").

b) Iodoacetic acid; interferes with glycolysis.

Previous observations (M/1,000 ; pH adjusted to 6.85-7.25): Brachet ('34) - no effect on frog egg in presence of air. Necrosis and death at end of neurulation in absence of air.

c) Phenylurethane; acts as a poison for dehydrogenases.

Previous observations (M/1,000): Brachet ('34) - development stops immediately when eggs are put in two hours post fertilization. Effects are less severe the older the embryo is at time of subjection.

In setting up the experiment, eggs were put into control, DBSA, KCN and Iodoacetate solutions one hour post fertilization. However they were put into phenylurethane at later stages (a, late cleavage and b, yolk plug) in order that development might proceed somewhat before the onset of effects.

The results with KCN were surprising in that they were not at all like those of Brachet or Bellamy. On the contrary, the animals developed at the same rate as the controls and were normal in all respects.

The reasons for the lack of effects are not clear. It may be that a stronger concentration is needed under the conditions of these experiments, in order to duplicate

the effects of previous workers. This is in conformity with certain observations, made in another connection, by R.W. Briggs * who also found that embryos of Rana pipiens, when subjected to various concentrations of KCN, showed much less severe effects than those described by Bellamy or Brachet.

The Iodoacetate had no effect on development, and the phenylurethane had only general effects (retardation, bent axis, etc.) but caused no degeneration whatsoever.

In any case, the experiment served only to some extent to answer the question originally posed, i.e. do metabolic poisons, when effective, cause the same type of alterations of development as DBSA ? However, judging from descriptions of Brachet and Bellamy, they do not. It might be added that even if the poisons and the DBSA were to cause identical changes in development under our experimental conditions, it would merit further investigation but it would by no means be conclusive evidence that the carcinogen is acting simply by means of straightforward interferrence with metabolism.

F. Salts and miscellaneous substances

This experiment was done in the specific hope of duplicating some of the results produced by Jenkinson ('06)

* Unpublished work

who studied the effects of 36 different substances (33 salts, cane sugar, dextrose and urea) on the development of the frog's egg (R. temporaria).

In certain solutions (cane sugar, $MgCl_2$, NaCl, NaBr and several others) he observed changes which, both in the gross and in section, appeared to be strikingly like those observed by us as a result of exposure of eggs to concentrations 2.13 and 4.26 of DBSA. Specifically he described what he termed "grey degeneration", which in cane sugar was first seen in the widely open medullary groove. The grey color was found to be due to a retreat of pigment from the surface to the interior of the superficial cells. The cells subsequently rounded up and broke away or were ejected from the epithelium. The degeneration always began in the neural, or medullary, groove and closure of the neural folds was specifically inhibited. *

In an attempt to duplicate these results (exp. #26) eggs were reared in solutions of(a) NaCl 0.625% ; (b) NaBr 1.09% ; (c)$MgCl_2$ 0.77% ; and(d) cane sugar 6.6%, the concentrations being those used by Jenkinson, all of whose solutions were isotonic with 0.625% NaCl.

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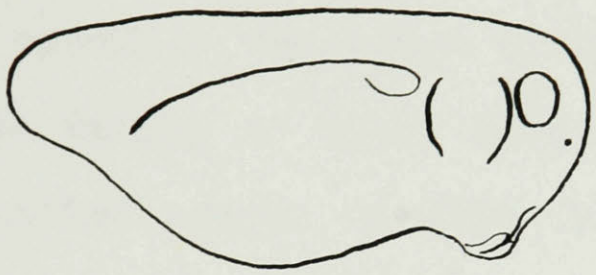
* According to Jenkinson this type of degeneration in frog's eggs and embryos was first described by Roux (1885) who called it "framboisia embryonalis finalis".

In NaCl and NaBr the eggs became shrunken and distorted, the yolk in most cases failed to cleave and development ceased during mid- or late cleavage. In cane sugar there were some attempts at gastrulation, but the eggs all died without the formation of a well defined blastopore. There was some "grey degeneration" but it was of cells scattered over the entire animal pole, and was not localized or initiated at the presumptive dorsal lip region.

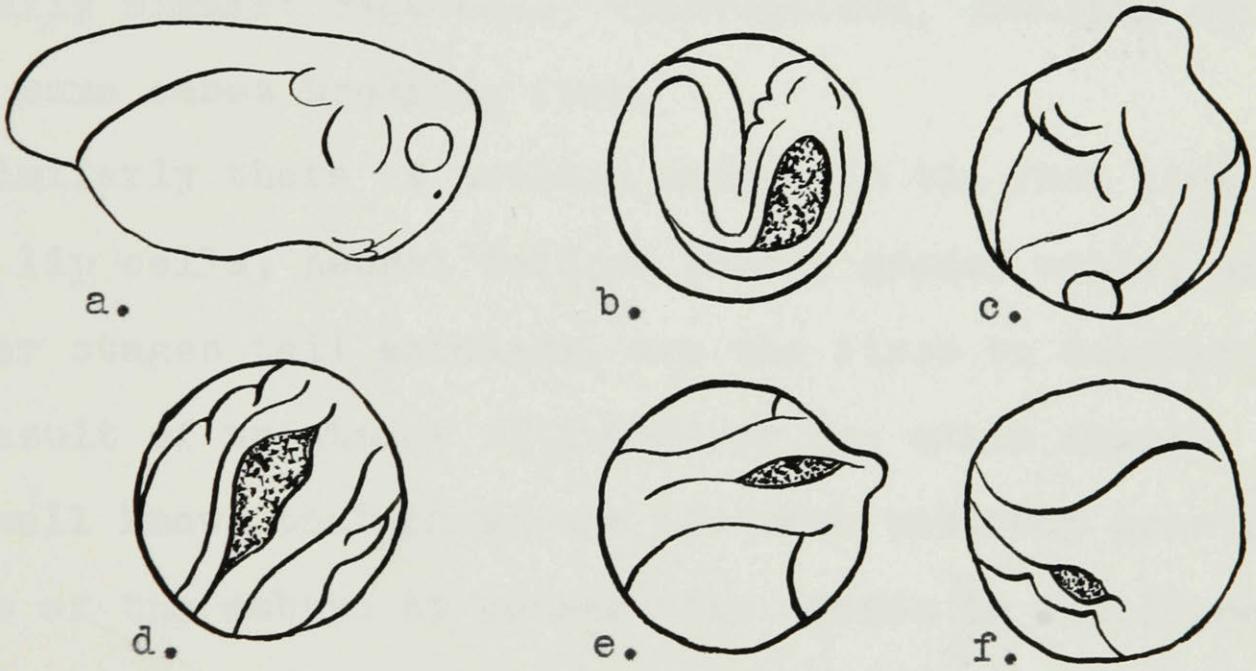
The $MgCl_2$ solution was the only one in which our results were in any way comparable with those of Jenkinson. However degeneration occurred in only 6 animals out of 20, and all of the animals with degeneration showed accompanying abnormalities, such as are shown in figure 27.

The primary effect of the salt appeared to be upon the vitelline membrane, preventing its expansion to allow for elongation of the embryo.

One animal hatched, but was extremely small for its morphological stage (fig. 27-a); some animals were doubled over (27-b), or parts of the anterior region forced themselves into the surrounding jelly as exovates (27, c and e). In extreme cases, some development proceeded but the embryos remained completely spherical (27-f).



control-90 hrs.



$MgCl_2$ embryos-90 hrs.

Fig. 27. Effects of $MgCl_2$ solution (0.77%) on early frog development. Typical abnormalities are shown. Animals a-f are arranged in approximate order of severity of effect. Stippling indicates degeneration. For further description see text. (Mag. 13.2 X. - camera lucida drawings).

Thus, in brief, the $MgCl_2$ produced a whole complex of changes of which neural groove degeneration may be considered only a manifestation, and not a dominant or even constant feature.

As regards the specific character of the degeneration, it is in neither case ($MgCl_2$ or DBSA) unique. There are, after all, only a limited number of ways in which a cell may die. In the case of yolk cells there is usually cytolysis and vacuolization; and in pigmented cells there is usually pigment recession, spherulation, swelling up, and in some cases breaking away.

Similarly there is nothing unique in the fact that dorsal lip cells, neural fold or neural groove cells, and in later stages tail ectoderm, are the first to degenerate as a result of treatment with DBSA or any other agent. It is well known that these are the most actively growing regions of the embryo at consecutive stages in development, and it has been repeatedly shown by Childs ('41) and others that such regions are the first to be affected by any injurious agent.

What is of interest, however, is that the DBSA, apparently unlike other agents, causes only degeneration of the most susceptible tissues, allowing development and differentiation of the rest of the animal to proceed for a period without any abnormality or effect other than retardation. To state it in other words, it is probable that any agent which

would cause neural fold degeneration comparable to that of concentration 4.26 DBSA, for example, would cause gross structural abnormalities as well, whereas the carcinogen does not. Thus in the specificity of its action, and in the 100% uniformity of the effects produced, it appears to differ from other agents.

Discussion

The extensive literature on the effect of carcinogens on mammals (see Hartwell '41 for tabular summary) is pertinent here only in two connections. Firstly, because certain workers have demonstrated that the specific water-soluble derivative of dibenzanthracene used by us, produces tumors in mice (Barry et al '35; Parsons '36; Burrows and Cook '36). Secondly there are numerous references to the fact that carcinogens inhibit body growth (Haddow '36; Haddow, Scott and Scott '37; Lee '37; White and White '39), and also inhibit the growth of spontaneous and induced tumors (Haddow '35; '38, a and b; Haddow and Robinson '37; '39; Badger et al '42). This evidence is in conformity with our observation on the retardative effect of DBSA on the growth rate of embryos.

Work on the effect of carcinogens on Amphibia is summarized in table 1, and is important in this connection mainly in that it shows that Amphibia are, to some extent at least, susceptible to tumor induction. (Work on Amphibian embryos will be discussed below).

The effects of carcinogens on tissue cultures, and on small free-living animals, however, are well worth analyzing in relation to the results observed by us, particularly in regard to effect on growth rate where reports are

Table 1: survey of reports on the effects of carcinogens on Amphibia

investigator	animal	substance	animals with tumors	notes
Briggs '40	154 frog tadpoles (R. pip.)	methylcholanthrene 0.2 mg/animal	3	out of 12 which retained implants.
Duran-Reynals '39	11 frogs (R. pip.)	3,4-benzpyrene	0	duration of exp. 20 days.
"	36 frogs (R. pip.)	20-methylcholanthrene	0	31 died in 1 month. Duration of exp. 4 months.
"	214 frogs (R.pipiens, R.clamitans, R.catesbiana	DB	0	131 died in 1 month. Duration of exp. 7 months.
"	18 newts (T. pyrrhogaster, T.viridescens	DB	0	all dead in 3 months.
Hellmich '28	1 salamander (Amb. tigrin.)	Sudan III	0	nodules which disappeared.
"	3 newts (T. cristatus)	"	1	a "preblastomatous process".
"	frogs	"	2	carcinomas.
Kinosita '37	toads	1,2-cyclopentenophenanthrene	0	---
"	"	DB	0	---
"	"	o-aminoazotoluene	0	---
"	"	azobenzene	0	---

Table 1. (cont.)

investigator	animal	substance	animals with tumors	notes
Kinosita '37	toads	p-dimethyl-aminoazo-benzene	0	---
"	"	"androstin" impure, chiefly testosterone	0	---
Koch, Schreiber & Schreiber '39	6 newts (T.cristatus T.taeniatus)	3,4-benzpyrene	5	duration of exp. 108 days.
Martella '35	newt	tar	--	no effect on regeneration.
Nakano '37	toad	3,4-benzpyrene	0	---
Shen '39	Triton embryos	Na-1,2,5,6-DBSA	--	induction.
Waddington '38	newt gastrulae	(various)	--	induction.
Waddington & D. Needham '35	newt gastrulae	"	--	induction.
Woerdeman '36	axolotyl gastrulae	1,2,5,6-DBSA	--	induction.

contradictory.

Creech ('39 and '40), using cultures of mouse fibroblasts, observed that 20-methylcholanthrene choleic acid (0.001mg/cc.) and 1,2,5,6-dibenzanthracene choleic acid (0.01mg/cc.) caused significant increase in cell proliferation as determined by mitotic counts and measurements of outgrowth, compared to control cultures (untreated, or treated with desoxycholic acid, phenanthrene choleic acid, or acenaphthene choleic acid). [This concentration of DB choleic acid has an actual concentration of 0.0015mg. DB per cc. and is thus equivalent to our 0.213mg./100cc. DBSA solution which caused no increase in growth rate in our experiments.]

In tenfold greater concentrations Creech found that both carcinogens caused definite retardation of growth, which is in agreement with our results.

No definite effect on growth was observed by

a) Bisceglie and Grazia ('36) using colloidal suspensions of 1,2-benzpyrene on cultures of chick embryo heart;

b) Larionow et al ('38) using a saturated solution of dibenzanthracene on fowl fibroblasts;

c) Timofeevsky et al ('39) using dibenzanthracene (1:1,000-1:5,000), methylcholanthrene, and 3,4-benzpyrene (1:500,000-1:1,000,000) on embryonic tissues of rat,

leucocytes of rabbit blood, and bone marrow of adult rats;

d) Magat et al ('37) who exposed mouse and fowl tissues to DB for two to eighteen days.

In contrast to these results Mauer ('38) and Earle and Voegtlin ('38 and '40) both observed definite retardation and toxic effects as a result of treating tissue cultures with carcinogens.

Mauer exposed chick fibroblasts to 3,4-benzpyrene, 1,2-benzanthracene, 1,2,5,6-DB, and 20-methylcholanthrene in concentrations of 1/40,000 and 1/400,000. Retardation and eventual cessation of growth occurred with both concentrations, although sooner in the stronger one. The degenerative changes observed were characterized by abnormal cell forms (enlargement and vacuolization of cells), and abnormalities in cell cleavage (multi-lobulated nuclei, chromosomal aberrations, and multipolar mitoses).

These findings agree with those of Earle and Voegtlin who used methylcholanthrene in concentrations ranging from 2.0 - 0.0002mg. per cc. of culture fluid, on cultures of rat and mouse fibroblasts. After a latent period of some days they observed a marked inhibition leading to complete or nearly complete cessation of growth, and accompanyied by varying degrees of cell destruction

(necrosis, rounding up, etc). The severity of the effect was roughly proportional to the concentration used, and there was never any evidence of growth stimulation even in the 0.0002mg/cc. concentration.

In their first report ('38) Earle and Voegtlin observed the cultures for only 54 days. In their subsequent work ('40) they extended their observations to 380 days after the addition of the carcinogen to the medium; (comparable doses of methylcholanthrene take 225-250 days to induce tumors in mice in vivo). After such a period of time they observed definite changes, increased lateral adhesion of cells, irregularity of cell size, etc. These changes were similar to those found in cultures of tumors induced by methylcholanthrene in vivo, and therefore led the authors to suggest that a malignant transformation might have occurred. However this has not yet been substantiated by injection of the culture material into animals. As to the main effects of the carcinogen - retardation and cell injury - the general nature of their findings is in complete agreement with our results.

(In regard to the question of the relation between carcinogenicity and effect on growth rate, it is obvious that many agents completely unrelated to carcinogens can

stimulate growth (for example, high temperature). Some investigators such as Wolbach ('37) have added that, in the case of carcinogens, there is no evidence to support the theory that the agents owe their carcinogenic property to direct stimulation of cell growth. Others have pointed out that stimulation of cell proliferation alone does not lead to neoplasia. Haddow ('36) even goes so far as to postulate that carcinogens act to produce irreversible variants of cell types through their growth inhibitory capacity.).

Various experiments on the effect of carcinogens on invertebrates and plants are listed in tables 2 and 3, respectively. It will be seen that in the majority of cases a stimulative effect, with no evidence of degeneration, has been observed, - the only significant exception being the work of Reiss, in which some degenerative changes were noted.

Since the majority of the carcinogens used in these experiments were insoluble or only very slightly soluble in water, it is possible that the effective concentrations were very minute. If this was the case, it may explain the difference in results between these experiments and ours, in which the effective doses, by comparison, would have been very large.

Table 2: experiments on the effect of carcinogens on invertebrates

animal	investigator	carcinogen	conc.	results
PROTOZOAN				
Paramecia	Spencer & Melroy '40b	MC *	0.001mg. /cc.	exposure over a yr. increased survival value.
Paramecia	Mottram '39; '40	3,4-BP *	1/500,000 1/1000,000	increased growth rate. Some abnormal forms.
Paramecia	Wolman '39	MC, BP, DB. colloidal.	optimal conc.'s: MC. 1:3,000,000 BP. 1:2,000,000 DB. 1: 200,000	proliferative effect.
Didinium nasutum	Calkins '16	(normal & cancer tissue)	--	both stimulate. Cancer tissue has lethal effect.
Eberthella typhi	Spencer & Melroy '40b	MC	1/1000,000	immediate stim. of rate of cell division.
Escherichia communior	Goldstein '37	colloidal DB & MC	--	increase in no. of 50% after 8-9 hrs.
"	Hopper & Clapp '39	DB & MC	0.3mg/cc.	increase in no. of cells in 5-15 hrs.
COELENTERATE				
Hydra	Tokin	"synthetic carc."	--	no effect on regeneration.
Obelia geniculata	Reiman & Hammett '35	DB	sat. sol. in sea H_2O	stimulation of growth.
"	Hammett & Reiman '35	MC	"	enhanced growth of anlagen.

* MC = Methylcholanthrene; BP=Benzpyrene.

Table 2, (cont.).

animal	investigator	carcinogen	conc.	results
PLATYHELMINTHE				
Planaria	Owen et al '38; '39	DB	aqueous sat.sol.	stimulates regen. of cut segments & reproduction of whole animal.
ANNELID				
Neries diversicolor	Thomas '31; '32	arsenic; indol; coal tar; B. Tume-faciens	--	1st 3: mild inflammatory reaction. Bacterium Tumefaciens: intense inflam. & large invasive "tumors".
ECHINODERM				
Sea urchin eggs	Reiss '24	tar	emulsion in sea H_2O	alteration of nuclei. Some degenerative changes.
"	Tchakhotin '38	(mono-bromo & mono-iodo Na-acetate	--	excessive proliferation of mesenchyme cells. Change considered "cancerization".
Arthropod				
Drosophila	Slizynski '36	tar	--	25% died. Remainder of P_1 normal. Decrease in no. of F_1 and F_2. Change in sex ratio.
PROTOCHORDATE				
Ascidian mentula	Thomas '32	arsenic; indol; coal tar; B. Tume-faciens	--	1st 3: mild inflammation in tunic. Bacterium Tumefaciens: large "tumor".

Table 3: experiments on the effect of carcinogens on plants

plant	investigator	carcinogen	results
Yeast	Cook, Hart & Joly '38; '39	DB	9×10^{-4} molar: maximum (50%) increase in proliferation. 4x that dose: inhibition.
"	Dodge & Dodge '37	MC; sat. MC-peptone-glucose solution	giant cells; increased differentiation. Increase of 1/3 in rate of fermentation & dry wt. of cultures.
"	Hollaender et al '39	MC; sat. sol. in physiological saline	stimulated growth in dark. Toxic effect in light (near ultra-violet radiations).
Fusarium lini	Wright & Anderson '38	DB; (H_2O-soluble oxidation products)	initial inhibition. Then increase in wt. of mycelium & increased utilization of glucose. (Parent hydrocarbon had no effect).
Allium cepa	Levine & Bergmann '36	coal tar; DB	cytoplasmic & nuclear changes. Inhibition of growth of root tips.
Pisum sativum	Owen et al '39	DB; aqueous sat. sol.	stimulates growth of rootlets.
Helianthus	Berthelot & Amoureux '37	DB; 1,2-BP; folliculin; indol acetic acid; suspensions	only plants treated with folliculin showed "neoplastic reaction". Plant treated with DB blackened, "suffered" & dried out.
Corn roots	Patton & Nebel '40	MC; DB; acenaphthene	lowered rate of elongation of root in vitro. Lowered rate of respiration. Increased size of nucleus.

In regard to the inductive capacity of carcinogens (see table 1) there is an interesting difference of interpretation. Waddington and D. Needham ('35)obtained neural tissue induction by implantation of two estrogenic and one carcinogenic (DB) hydrocarbon into newt gastrulae.

Waddington ('38) implanted various substances into the blastocoeles of newt gastrulae (approximately 0.02mg. of the substance in albumen per embryo, which is approximately 5.0mg. wet weight). Methylcholanthrene and Na-1,2,5,6-dibenzanthracene-endo-α,β-succinate showed high inductive capacity, as did some estrogenic compounds. However 3,4-benzpyrene, a potent carcinogen, showed only moderate inductive power. Controls, having only albumen implants, were used (though not conclusively) to establish the fact that the inductions were not due to injury or the presence of foreign material alone.

Shen ('39) implanted albumen containing Na-1,2,5,6-dibenzanthracene-endo-α,β-succinate into the blastocoeles of developing embryos of Triton alpestris. He found a dose of 0.0125 gamma to be optimum for neural tube induction, doses either below or above that being less effective.

(Abercrombie, working with chick embryos, brought about neural plate evocation by use of a variety of chemical

substances, including DB and DBSA).

Of the two possibilities, 1) that the carcinogens act directly as evocators, or 2) that the carcinogens act by liberating the embryo's own evocator, these workers of the English school appear to favor the first view. Their reasons are: that the dosages necessary for evocation are of the same order as those of certain other biologically active agents such as drugs, vitamins and hormones (which presumably act directly); there is a close chemical relationship between carcinogens and evocators (Needham '36); there is an optimal dose curve (Shen) rather than a greater effect with increased dosage as might be expected if the carcinogen acted by liberating a natural evocator through injury; pycnotic nuclei were not found to any greater extent at higher dosages than at low ones, which again suggests that injury and degeneration do not play a large part in the reaction (Shen).

In contrast, Woerdeman ('36), as a result of work on the inductive effect of 1,2,5,6-dibenzanthracene on axolotyl gastrulae, reached the conclusion that "inductive" or "organizing" substances "should be considered merely as stimulating substances, and that the real induction itself is performed by the host".

This interpretation would certainly seem to be more in conformity with all that is known about the mechanism of carcinogenesis and the "escape from organizing influences" which is a characteristic of cancer tissue. It would seem plausable to postulate that the carcinogen induces proliferation which the embryo, because of its strong "organizer" capacities, can bring under orderly control. But it seems somewhat paradoxical to postulate that substances, which are known to be carcinogenic, may actually operate as direct "organizers" of orderly growth.

In our own experiments there was only one instance in which the carcinogen had any suggestion of "inductive" action. Namely, in experiment 18, eggs which had been exposed to DBSA for one month in the female before ovulation, showed a suggestion of multiple blastopore formation. However since the embryos were definitely abnormal and development ceased shortly afterward, it is difficult to draw any conclusions as to the true nature of the effect.

In a more general way, however, our experiments definitely support Woerdeman's hypothesis that the carcinogens act only indirectly, in that one of the primary effects of the carcinogen was cell injury, and that

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In a more general way, however, our experiments definitely support Woerdeman's hypothesis that the carcinogens act only indirectly, in that one of the primary effects of the carcinogen was cell injury, and that

effect (contrary to Shen's observations) definitely increased with increase in dosage.

* * *

The apparent differential effect of the carcinogen on the development of form and of function is of some interest. As has been mentioned before, it was noted in several experiments that animals in concentration 2.13 (i.e. animals which survived but showed definite retardation) did not respond to mechanical stimulation even when, according to external appearances, they had reached or even passed the stage at which such response should become manifest.

Needham ('33) has reviewed evidence for such dissociation of fundamental ontogenetic processes. DuShane and Hutchinson ('41) have brought about differential development of form and behaviour in Amblystoma embryos by subjection to low temperatures, but this is perhaps the first time that such an effect has been brought about by means of a carcinogen.

* * *

One final point may be mentioned, namely the possibilities for further experimentation suggested by the present

results.

Various control experiments have indicated that the effects observed are due specifically to the carcinogenic properties of the DBSA. If such is the case (which may be tested by using a water-soluble derivative of some other known carcinogen), then the experimental set-up here described would be in some respects ideal for the testing of compounds for carcinogenic activity, and for determining relative potency of various carcinogenic agents.

The limitation of the procedure would be the fact that substances used would be restricted to those which could be converted to water-soluble forms.

The advantages of the procedure would be that the reaction of the embryos is clear-cut and uniform; the stage at which degeneration begins acts as an index of the potency of the agent; and finally, results are achieved in a matter of days rather than weeks or months as is the case when using mice as test material.

Summary and Conclusions

Eggs of the frog, Rana pipiens, were reared in solutions of Na-1,2,5,6-dibenzanthracene-9,10-endo-α,β-succinate, exposure beginning one hour after fertilization. The concentrations used were 0.02; 0.213; 2.13; 4.26; and 10.0mg. per 100cc.

The effects observed were:

1) Degeneration - beginning at the lips of the blastopore in concentration 10.0, and along the neural folds in concentration 4.26 (and in some cases concentration 2.13).

2) Failure of neural fold fusion in concentration 4.26 (and in some cases 2.13) without any other accompanying morphological defect or abnormality.

3) Retardation of growth rate of embryos in the three stronger concentrations, degree of retardation being proportional to the concentration.

4) Apparent differential retardation of development of form and behavior.

Subsidiary experiments indicated that

1) There is no long latent period involved. The carcinogen first becomes effective shortly after the initiation of gastrulation, at which time retardation

becomes grossly observable in the stronger concentrations.

2) Under the conditions of these experiments, the carcinogen is not "used up" in the process of exerting its effect upon a group of eggs.

3) Neither keeping nor using the carcinogen in the dark in any way lessens or alters its activity.

4) The exposure of older tadpoles (10 mm.) to a 4 mg./100cc. solution results in extensive injury (necrosis and hemorrhage) to tail fin tissue, and also causes papilloma-like growths within a few days.

Control experiments give evidence that

1) The capacity of the carcinogen to lower surface tension is not responsible for the effects observed.

2) The Na-succinate part of the molecule is not responsible for the effects observed.

3) A related non-carcinogen, anthracene-9,10-endo-α,β-succinate, used either alone or in combination with a surface tension lowering agent, has no effect upon development of the embryos.

These three observations, taken together, suggest that the effects brought about by the 1,2,5,6-dibenz-anthracene derivative may be due specifically to its carcinogenic properties.

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PLATES 1-5

These supplementary pictures (which show, in terms of photographs what is shown in figure 9 by camera lucida drawings) illustrate the effects observed in the "basic experiments", i.e. those experiments in which exposure of the eggs to solutions of the carcinogen was continuous, beginning at one hour post fertilization.

The magnification of all pictures is approximately 14 x.

PLATE 1

25 hours post fertilization - showing that all embryos are at approximately the same stage of development.

Fig. 28. Control.

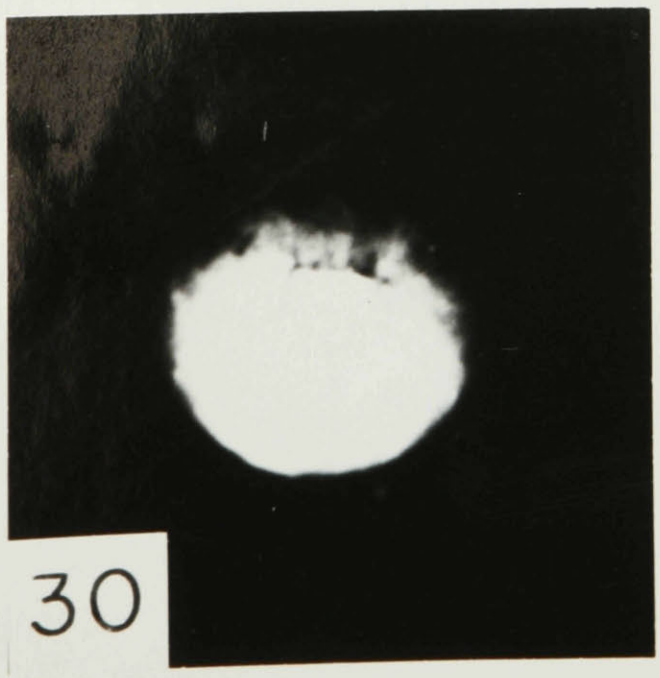
Fig. 29. Conc. 2.13.

Fig. 30. Conc. 10.0.

PLATE 1

28

29



30

PLATE 2

40-46 hours post fertilization, showing first evidence of the retardative effect of the carcinogen.

Fig. 31. Control.

Fig. 32. Conc. 0.02.

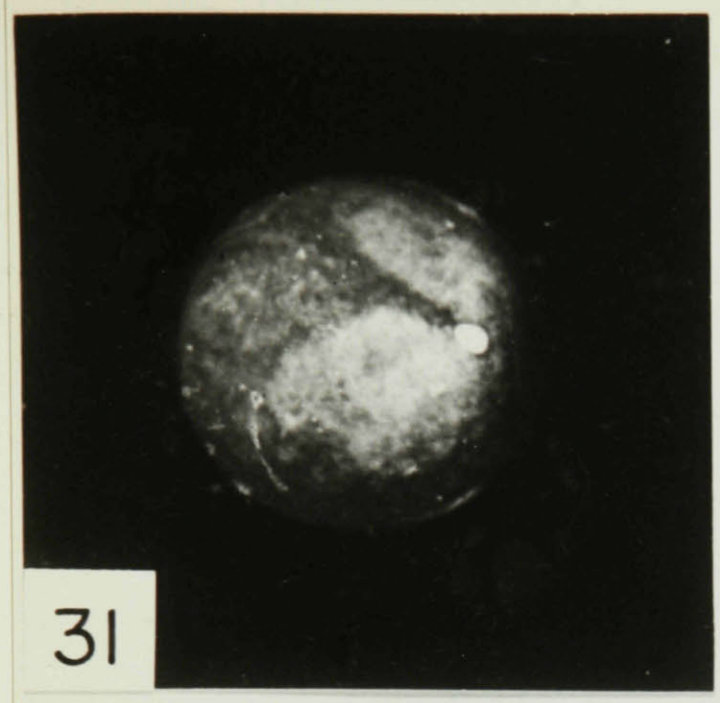
Fig. 33. Conc. 0.213.

Fig. 34. Conc. 2.13.

Fig. 35. Conc. 4.26.

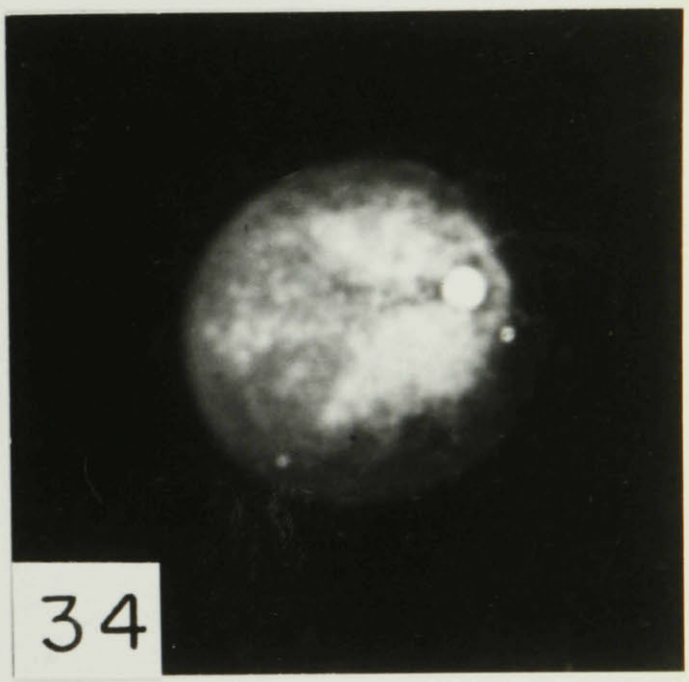
Fig. 36. Conc. 10.0.

PLATE 2


31


32


33


34


35

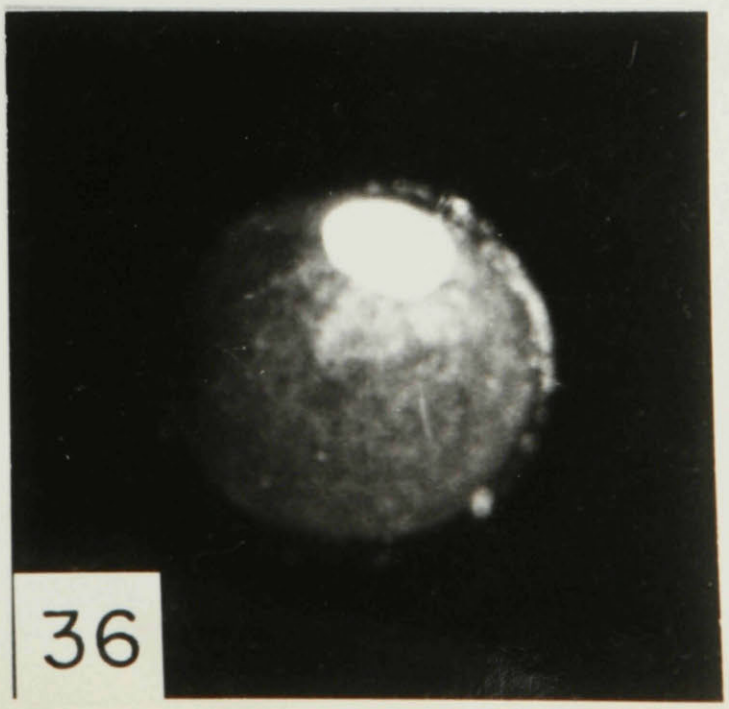

36

PLATE 3

54-58 hours post fertilization, showing stages in the degeneration of the embryos in concentration 10.0, with a comparable control.

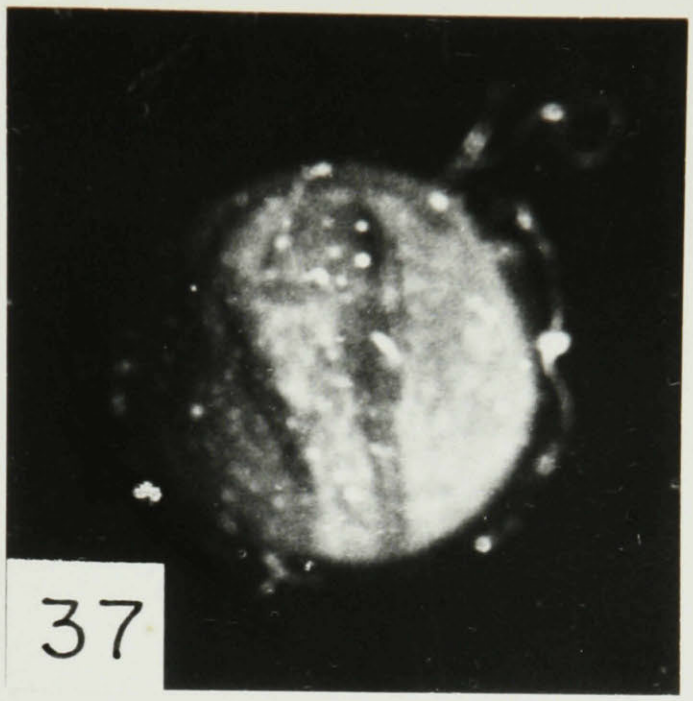
Fig. 37. Control, 54 hours, from which the degree of retardation of the 10.0 embryos may be judged.

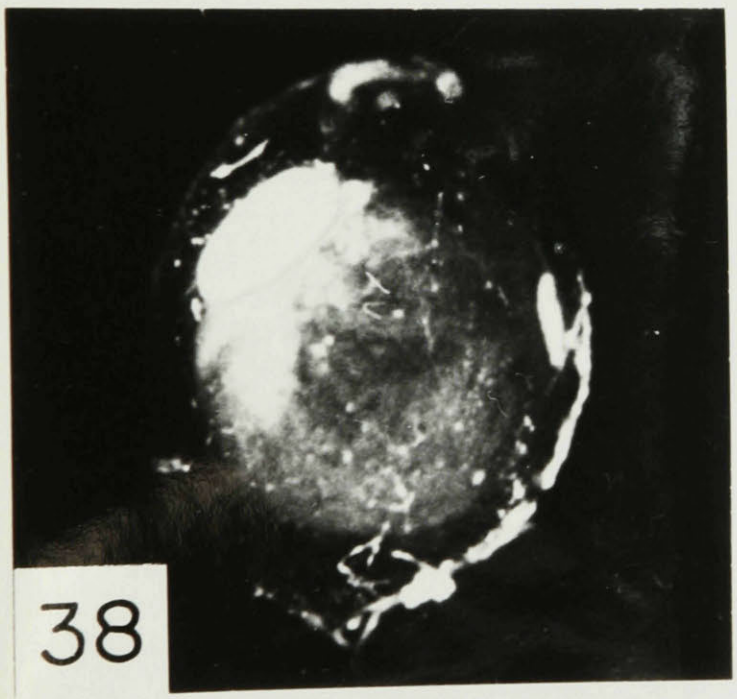
Fig. 38. Conc. 10.0; earliest stage in degeneration. A few pale swollen cells may be seen at the lip of the blastopore.

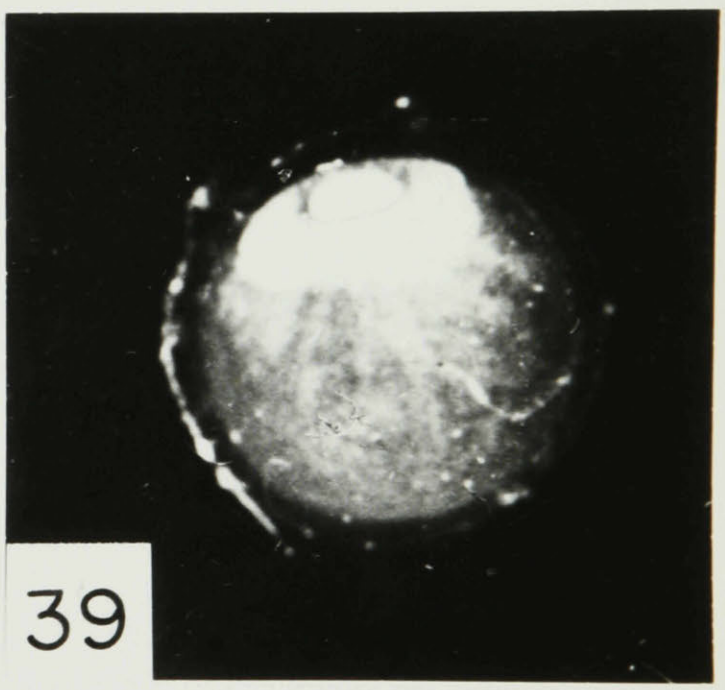
Fig. 39. Conc. 10.0. Degeneration has spread, forming a small "collar" of degenerated cells around the persistent yolk plug.

Fig. 40. Conc. 10.0. The "collar" has increased in size with the spread of the degeneration back over the surface of the egg.

PLATE 3







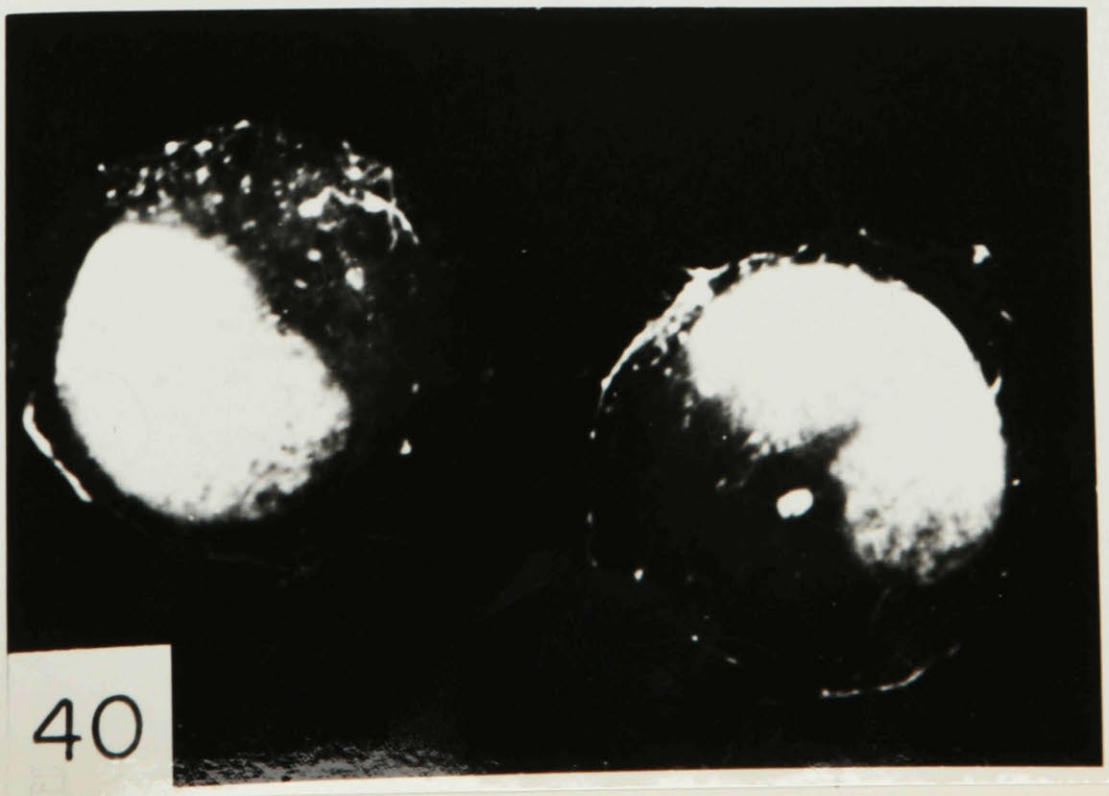


PLATE 4

70 hours post fertilization, showing retardative effect; beginning of degeneration in concentration 4.26; and complete degeneration in concentration 10.0.

Fig. 41. Control. Stage 16, neural folds fused.

Fig. 42. Conc. 0.02. Like controls.

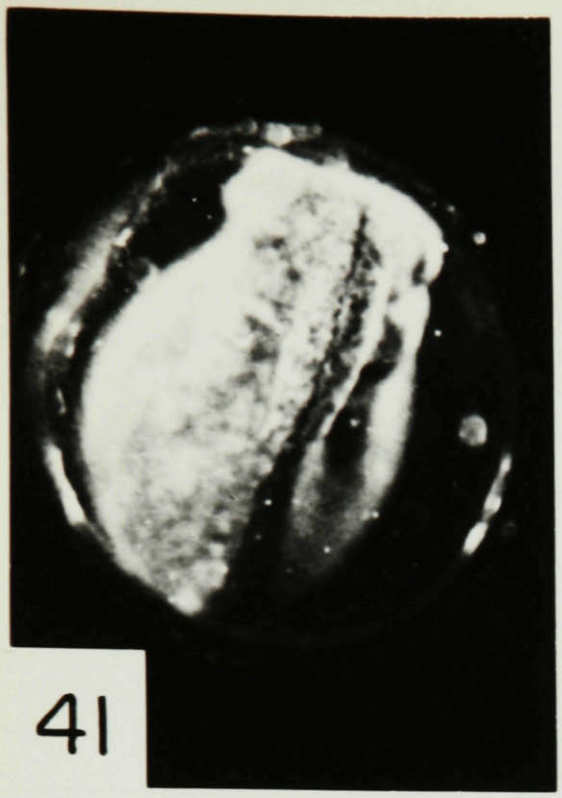
Fig. 43. Conc. 0.213. Slight retard (not always evident in this concentration). Neural folds not completely fused.

Fig. 44. Conc. 2.13. Definite retardation. The neural folds are close but not touching. Approximately stage 14 3/4.

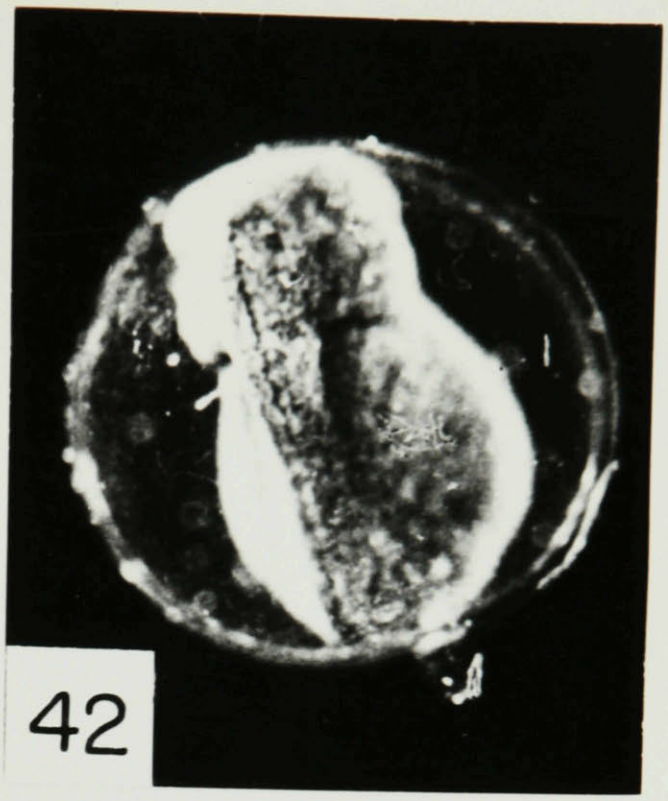
Fig. 45. Conc. 4.26. Retardation and earliest stage of degeneration. A few pale, swollen cells may be seen along the ridges of the neural folds (neural crest region).

Fig. 46. Conc. 10.0. Complete degeneration. Swelling of the egg is due in part to swelling of individual cells. Development stopped at approximately stage 12.

PLATE 4

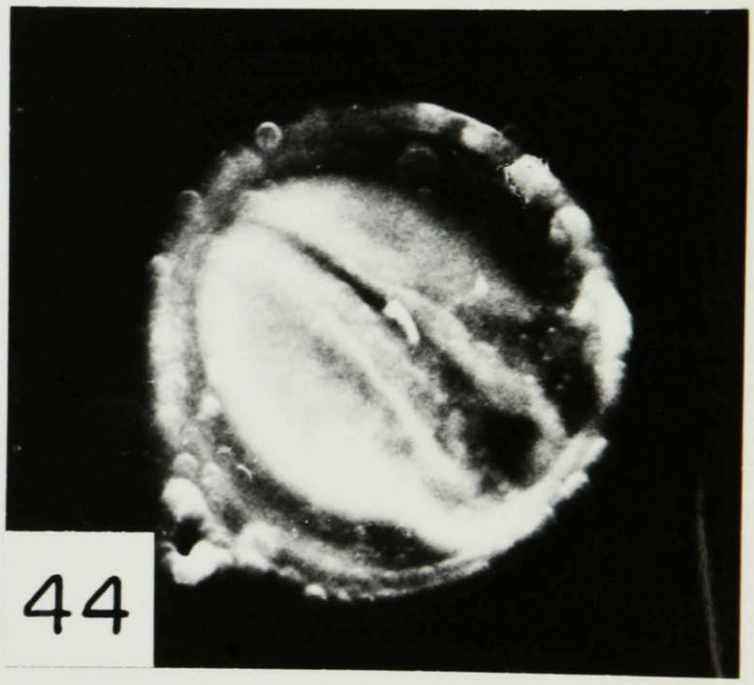


41



42

43



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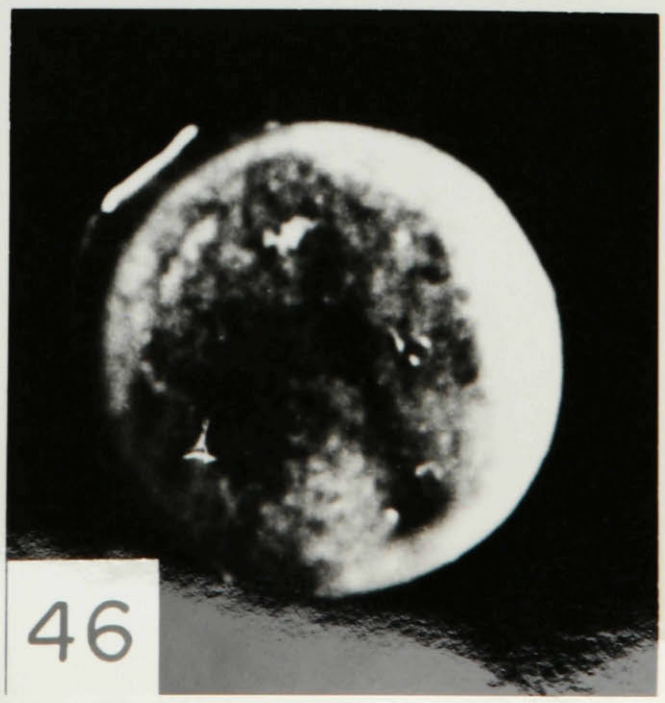

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PLATE 5

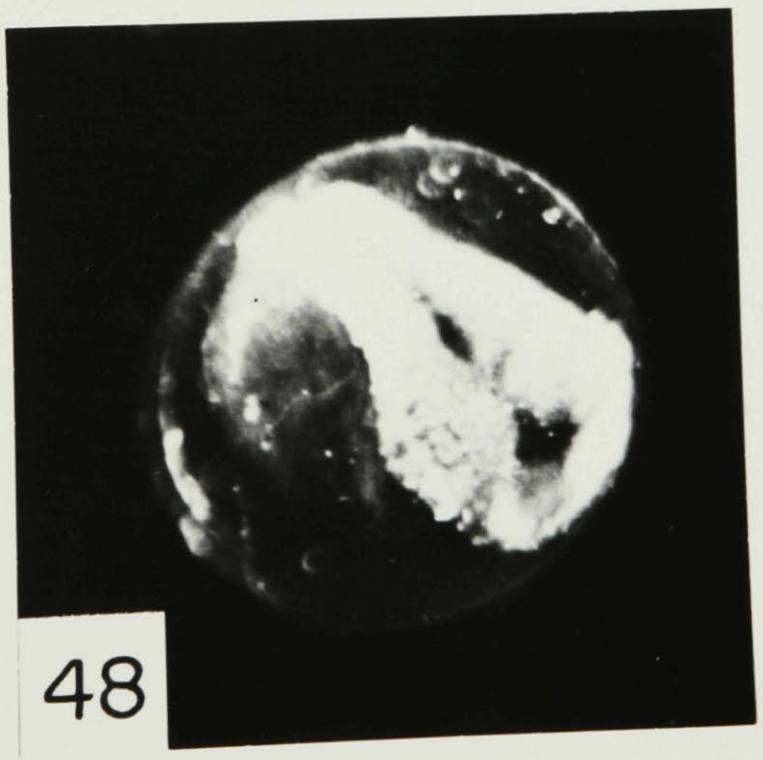
85-90 hours post fertilization, showing stages of degeneration of embryos in concentration 4.26.

Fig. 47. Conc. 4.26. Early degeneration. Pigment recession of individual cells along the neural crest region of the neural folds may be seen. Posteriorly, in each embryo, cells covering the entire width of the neural folds have become affected.

Fig. 48. Conc. 4.26. Complete degeneration of neural folds. It is only after this condition has been attained that degeneration spreads secondarily over the body ectoderm, causing death of the embryos at approximately stage $14\frac{1}{2}$.

PLATE 5

47



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Appendix I. Summary of experiments

Exp.#	total no. eggs	purpose	conditions*	notes
1	104	cleavage & later development	0.213; 2.13 4.26	eggs unmixed temp. uncontrolled
2	200	"	0.02; 0.2; 2.13; 4.26	"
3	150	controls, to check variation	--	"
4	115	"	--	eggs mixed.
5	200	"	--	"
6	200	cleavage and later dev.	0.2; 0.02; 2.13; 4.26	questionable eggs,
7, a&b	280	"	"	--
8,a,b&c	250	"	0.02; 0.2; 2.13; 4.26; 10.0	--
9	240	"	0.2; 2.13; 4.26; 10.0	--
10	240	cleavage & later dev. including growth measurements	0.2; 2.13 0.02; 4.26; 10.0 (2 controls)	thermostat. Temp. controlled from here on.
11	40	post neurula development	0.2; 2.13 4.26	animals put into solutions at stage 16.
12	40	effect on later development	4.0	R. sylvatica tadpoles. Put in when 10 mm.
13	100	effect of lowered surface tension	Aerosol O.T. 0.2mg/100cc.	--

* numbers indicate concentrations; mg. per 100 cc.

Appendix I (cont.)

Exp.#	total no. eggs	purpose	conditions	notes
14	25 *	effect of Na-succinate	Na-succinate equimolar with 10.0DBSA	--
15,a&b	175 120	effect of use on solution	"used" solutions from exp. #16	"used" and new solutions made at same time. Thus originally same age & conc.
16	245	repeat; effect on cleavage & later dev. Photographs.	0.02; 0.2; 2.13; 4.26; 10.0 (2 controls)	--
17,a&b	150 140	effect of ageing of solutions	unused solutions kept in icebox & at room temp.	icebox (4° C.) causes ppt. & consequent weakening of 4.26 and 10.0.
18, a b,1,2,3	90 450	injection of DBSA in vivo	eggs exposed before fert. 4 days; 8 days 1 month	--
19,a,b,c	196	effect on cleavage	0.2; 2.13; 10.0	only two dishes observed at once.
20	120	effect of metabolic poisons	KCN; M/10,000 Iodoacetic acid; M/1,000 (pH 7.4) Phenylurethane M/1,000	--
21	90	effect of related non-carcinogen: anthracene	anthracene SA (7.47) equimolar with 10.0 DBSA	--

* Control and 10.0 eggs for this experiment are included with a previously listed experiment with which this one was run concurrently.

Appendix I (cont.)

Exp.#	total no. eggs	purpose	conditions	notes
22	72	effect of fert-ilization in DBSA	fert. & kept in 1/10 Ring.; Fert. & kept in 10.0 DBSA; Fert in R,put in DBSA 18 min. post fert.; Fert in DBSA, put in R. 2 hrs. post fert.	--
23, a&b	125 75	effect of light on activity of DBSA	kept in light & used in light; Kept in light & used in dark; Kept in dark & used in light; Kept in dark & used in dark.	--
24, a	35 *	attempt to det-ermine time of action of DBSA	eggs in DBSA 1 hr. post fert. Put in 1/10 Ringer's 25 hrs. p.f. while still like controls	--
24, b	25 *	"	eggs reared in 1/10 Ringer's. Put in DBSA 25 hrs. p.fert.	--
25	35 *	effect of anthracene at lowered surface tension	anthracene SA equimolar to 10.0 DBSA, diluted with 0.2 Aerosol O.T.	--
26	80 *	effect of misc. substances	NaCl; 0.625%; NaBr 1.09%; $MgCl_2$ 0.77%; cane sugar 6.6%	(% = grams per 100 cc.).

Total: 4407

* Control and 10.0 eggs for this experiment are included with a previously listed experiment, with which this one was run concurrently.

Note: 1 control (1/10 Ringer's) was run with each exp. unless otherwise noted. A 10.0 DBSA was run with each exp. involving a substance other than fresh, unused DBSA.

Appendix II. The apparatus used for making growth measurements of embryos.

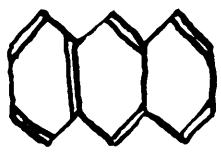
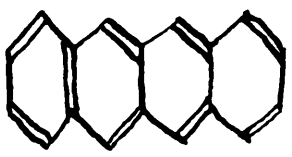
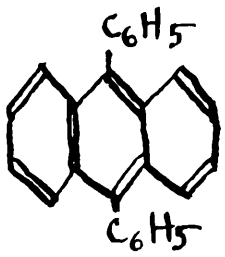
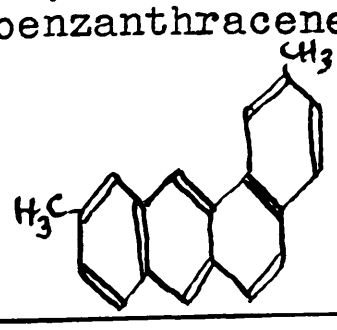
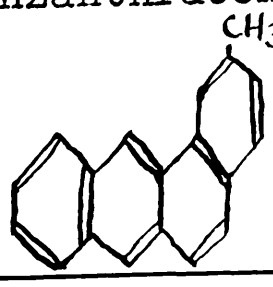
The "embryometer" consisted of a stationary microscope with a cross-hair eye-piece and a movable stage. The stage scale was in millimeters with a vernier reading to 0.1 mm.. The stage moved on a screw of 1 mm. pitch, the head of the screw being accurately graduated in hundredths, allowing readings to 0.01 mm., and estimates to 0.005 mm..

All parts of the mm. scale were calibrated with a 2 mm. stage micrometer, giving values from 1.995 to 2.005. Thus the error was ± 0.2% for measurements of a length of 2 mm..

The apparatus was also checked by measuring a preserved embryo 20 times, the animal being readjusted between each set of five readings. Result: 5.40 mm. ± 0.025 mm., giving an accuracy of ± 0.5%.

Animals were measured by focusing the cross-hairs first on one end (reading) and then moving the stage until the other end came under the cross-hairs (reading). The animals were anesthetized in MS. 222 (Rothlin '32), and when necessary, were placed on velvet to prevent movement during measurements.

Appendix III. Non-carcinogenic substances which are related to 1,2,5,6-dibenzanthracene, and of which the endo-succinate derivative can be formed.

substance	evidence of tumor	evidence of non-carcinogenicity	endo-succinate
Anthracene 	--	Haddow '36 Cook et al '37 Kennaway & Heiger '30 (Warren '39 - "none reported") (Hartwell '41, cites 12 additional workers who confirm non-carc.)	Warren '39 Bachmann & Kloetzel '38
Naphthacene 	--	Barry et al '35 Kennaway & Heiger '30 (Warren '39 - "none reported").	Warren '39
9,10-diphenyl-anthracene 	--	Wolman '39 (used it on paramecia. States that it is non-carc. No reference given). Velluz '38	Bachmann & Kloetzel '38
2',7-dimethyl-1,2-benzanthracene 	--	Barry et al '35 (Warren '39 -"none reported").	Warren '39
2'-methyl-1,2-benzanthracene 	--	Barry et al '35 (Warren '39 - "none reported").	Warren '39