BEAUFORTIA

Inhibition of the Processes of Growth and Differentiation in the Embryonic Development of the Axolotl (Ambystoma mexicanum) *)

by

ANTH. STOLK

(Histological Laboratory, Free University, Amsterdam)

1. INTRODUCTION.

The problem of the retardation of the processes of growth and differentiation is certainly as important as the processes of growth and differentiation themselves. It is striking, therefore, that whereas the analysis of growth has been carried out for a considerable period of time already, the analysis of inhibition was only commenced a few decades ago. It has to be admitted that WIESNER (1894) succeeded in demonstrating the presence of a substance retarding germination in the slime of the mistletoe (*Viscum album*), but this remained a solitary observation for some time.

About 1920 a series of important publications appeared which deal with inhibiting substances. OPPENHEIMER (1922) discovered a substance of this kind in the fruit pulp of ripe tomatoes, REINHARD (1933) found one in tomato juice, KÖCKEMANN (1934) some in other pulpy fruits such as apples, pears, quinces and tomatoes, LEHMANN (1937) one in the exocarp of buckwheat, RUGE (1939) some in the fruits of *Helianthus annuus* and *Avena sativa*, FRÖSCHEL (1939, 1940) one in *Beta*, STOLK (1952, 1953a) some in the roots of *Fuchsia hybrida* and *Pelargonium zonale* and in the roots of *Allium Cepa*.

These inhibiting substances show a difference in behaviour towards the temperature. The substances reported by OPPENHEIMER (1922), LEHMANN (1937) and STOLK (1952) appeared to be thermolabile, those found by REINHARD (1933), KÖCKEMANN (1934), FRÖSCHEL (1939, 1940) and STOLK (1953a) thermostabile. Also in their chemical behaviour these substances show some differences. Whereas the substance described by KÖCKEMANN is soluble in ether, this is not the case with the substance obtained from tomatoes by OPPENHEIMER.

These substances will not be discussed here; for further details the reader is referred to the publications by the author (STOLK, 1952, 1953a). As, however, the present investigation is a continuation of the author's

[51]

work on the substances retarding root growth in *Fuchsia, Pelargonium* and *Allium,* a short survey is given here :

1. *Pelargonium* cuttings excrete a substance inhibiting the growth of *Pelargonium* roots and *Fuchsia* cuttings produce a substance inhibiting the growth of *Fuchsia* roots : auto-inhibition.
2. The inhibiting substance from *Pelargonium* also inhibits *Fuchsia* roots and acts aspecifically : inhibition sensu stricto.
3. The inhibiting substance from *Fuchsia* is weaker in its action than the one from *Pelargonium.*
4. Both the *Fuchsia* and *Pelargonium* inhibitors are thermolabile, the inhibiting capacities get lost after a treatment at 100° C.
5. If the inhibition has not been lasting for too long a period, it can be stopped by a transfer of the cuttings to fresh water. The inhibition is therefore a reversible process.
6. The inhibiting substance from *Fuchsia* cuttings has no perceptible influence on the formation of lateral shoots.
7. *Allium* roots excrete a substance retarding the growth of *Allium* roots : auto-inhibition.
8. Also in *Allium* the inhibition can be stopped by a transfer of the inhibited root to fresh water and is, therefore, a reversible one.
9. The root-inhibiting substance of *Allium* is thermostabile. Unlike the root-inhibiting substances of *Fuchsia* and *Pelargonium* the growth-retarding action is not lost by a heating at 100° C.
10. The root-inhibiting substance of *Allium* can be concentrated by evaporation both at a moderate temperature (15—20° C.) and at a higher temperature (100° C.).
11. The root-inhibiting substance of *Allium* does not maintain its inhibiting action indefinitely. In dependence on time inactivation occurs.
12. Also the aequous extract of *Allium* roots retards root growth and must therefore contain an inhibiting substance.
13. The inhibiting action of the aequous extract of *Allium* roots is not lost after heating it at 100°C. : thermostability of the extract of *Allium* roots.
14. The inhibiting action in *Allium* could be represented in a diagram. Most probably three phases have to be distinguished in this process, viz :
    a. a phase of inhibition in which the inhibiting substance gradually decreases the speed of growth of the roots ;
    b. a phase of equilibrium in which the inhibiting substance is not capable of reducing the speed of growth any further, so that the speed of growth remains practically constant ;
    c. a phase of recovery in which the roots show an increased speed of growth again.

The obvious question was whether or not certain substances, other than hormones, could be found in animals which are comparable to those retarding germination or root growth.

DALCQ, FRÖSCHEL and BRANDES (1942) succeeded in causing retardation of the development of the eggs of *Rana fusca* by means of an aequous extract of *Viscum album* fruits. Occasionally a toxic effect of this extract was observed : spina bifida, edema, plurilocular auditory vesicles, abnormal development of the organs of smell and sight, several

cases of abnormal bending of body and tail. On the strength of the histological examination these authors think it probable that the inhibiting action is related to the "dynamisme cyto-nucléaire".

Although the inhibition of *Rana fusca* eggs was only of a temporary nature (after 5 to 6 days there was no longer a difference between embryos reared in water containing the extract and embryos reared in tap water) we decided to carry out inhibition experiments also with Amphibian eggs. As a test object eggs of the Axolotl (*Ambystoma mexicanum*) were chosen. In contradistinction to DALCQ, FRÖSCHEL and BRANDES (1942) we did not intend to study the inhibition by certain substances, but only the self-inhibition eventually occurring in Amphibian eggs, i.e. the retardation of growth and differentiation of the egg by a substance produced by the egg itself.

The excretion of substances into the water by aquatic animals has been frequently shown. The pertaining literature need not be extensively cited and only a few publications will be mentioned.

JASKI (1939a, 1939b) for instance showed that sexually mature, male specimens of the viviparous Cyprinodont *Lebistes reticulatus* (PETERS), the guppy or millionfish, excrete a substance into the water, which is supposed to be taken up by the ovarium of the virgin female via gills, blood and pituitary gland and cause the oblique position (the so-called "elevation position") of the female, in which the spinal column forms an angle of 30 to 70 degrees with the horizontal plane. This is of course very favourable for a subsequent copulation, the sperm being brought into the oviduct via the female genital pore by means of the anal fin which is transformed into a long tube (gonopodium). In view of the function of this substance in copulation JASKI called it copulin.

According to DE GROOT and DUYVENÉ DE WIT (1949) copulin is also present in the Cyprinid *Rhodeus amarus* (BLOCH), the bitterling. The substance is excreted into the water by the males and causes the growth of the ovipositor in the female. Under the influence of a constant strong illumination the production of copulin appeared to decrease. The question of copulin will not be discussed here. For further details the reader is referred to the pertaining publications and to STOLK (1950a).
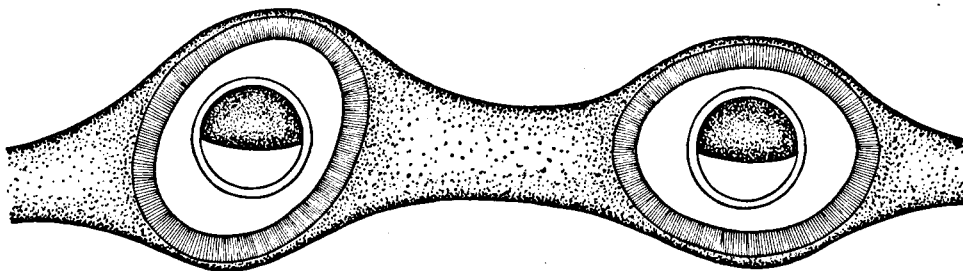

FIG. 1. Diagram of *Ambystoma* eggs.
    The eggs, which are enclosed in thin yolk membranes, lie in transparant capsules filled with a liquid. The capsules are connected by a gelatinous substance, so that a string of eggs is formed.

2. MATERIAL AND METHOD.

    As experimental material the eggs of *Ambystoma mexicanum* were

used. The eggs, which are enclosed in thin yolk membranes, lie in transparant capsules filled with a liquid (fig. 1). The capsules are connected by a gelatinous substance, so that a string of eggs is formed. The capsule liquid appears to contain a number of salts.

It was assumed that the capsule must on the one hand fence off the interior and on the other hand have a certain permeability (e.g. for the required oxygen). If the *Ambystoma* egg would eventually excrete some inhibiting substance, this inhibiting substance must reach a higher concentration when the egg has only a small amount of water at its disposal than when a larger amount of water is available. The same holds for an egg with intact capsule and for an egg from which the capsule has been removed, respectively.

In the first series of experiments *Ambystoma* eggs of the same age (stage of the yolk plug) were placed in small containers with very little water and an other lot in large containers with a large quantity of water. Fig. 2 gives a diagrammatic representation of the experimental set-up. A small glass container was placed inside a large one and both were filled up with so much tap water to just cover the eggs. The external conditions (such as temperature and illumination) were perfectly identical. In order to avoid too rapid a development of the eggs — which was undesirable in connection with the mutual comparison — the experiments were carried
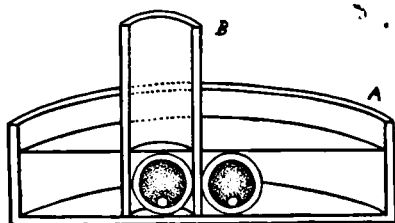

FIG. 2. Diagram of the inhibition experiment with *Ambystoma* eggs.
    A = large glass container (wide and low).
    B = small glass container (narrow and high).
    In the large container as well as in the small container an egg (in the stage of the yolk plug) is placed, as indicated in the figure.
    The gelatinous substance has been removed, but the capsules have been left intact. The yolk membrane is not indicated in the figure. The containers were filled up with so much tap water to just cover the eggs.

out in a room with a fairly low room temperature. The growth and differentiation of these eggs were studied, the differences only being attributable to the development in different quantities of water.

In the second series of experiments the capsules were removed from half the amount of test eggs of the same age, using two pairs of finely pointed watchmakers' tweezers. The yolk membrane was left intact, however. Both the lots of eggs with and without capsules were reared in Holtfreter's solution under identical external conditions. This solution corresponds with the liquid in the capsule as far as its composition is concerned. Also of these eggs the growth and differentiation were studied.

In these experiments we started from eggs in the stage of the yolk plug, in the neurula stage and in the stage of the tail bud.

The following criteria were used to estimate growth and differentiation :
a. closing of the neural folds ;
b. pigmentation (pigmentation of the belly, band-like pigmentation of fin fringe occurrence of yellow pigment cells);
c. length and ramification of the gills ;
d. development of the eyes ;
e. hatching of the embryos ;
f. head-tail length of the embryos.

Finally, it should be mentioned that the experiments were carried out during the months February and March.

3. DISCUSSION OF EXPERIMENTAL RESULTS.

From the considerable number of experiments forming the first series, two are selected and represented in tables 1 and 2 and in fig. 3. In both experiments we started from eggs in the stage of the yolk plug with intact capsules. Only the amounts of rearing water were different.

The tables shows that the various phenomena used as criteria appear almost simultaneously in the experiments with little water and in those

TABLE 1. Inhibition experiment with *Ambystoma* eggs in the stage of the yolk plug. The eggs, both having an intact capsule, were placed in a small glass container with little water and in a large glass container with a large quantity of water, respectively (see fig. 2). The growth and differentiation of the egg, developing in little water are considerably retarded.

Date	Time in days	Egg with capsule in the stage of the yolk plug in small container with little water	Egg with capsule in the stage of the yolk plug in large container with a large quantity of water
February 23	0	Stage of the yolk plug	Stage of the yolk plug
March 13	18	Embryo in the stage of the tail bud ; short gill primordia ; large, slightly pigmented yolk sac	Embryo in the stage of the tail bud ; long gill primordia ; small yolk sac showing abundant pigmentation
March 14	19	Small eyes ; no band-like pigment zone in fin fringe	Large eyes ; commencement of formation of band-like pigment zone in fin fringe
March 17	22	Small eyes ; no pigmentation of fin fringe ; hardly any yellow pigment cells.	Very large eyes ; band-like pigment zone in fin fringe ; yellow pigment cells
March 18	23	Small eyes ; little pigmentation ; no band-like pigment zone in fin fringe ; few yellow pigment cells ; head-tail length 11.8 mm	Very large eyes ; abundant pigmentation ; yellow pigment cells ; head-tail length 14.8 mm ; gills with well-developed ramifications ; early embryonal stage

TABLE 2. Inhibition experiment with *Ambystoma* eggs in the stage of the yolk plug. The eggs, both having an intact capsule, were placed in a small glass container with little water and in a large glass container with a large quantity of water, respectively (see fig. 2). The growth and differentiation of the egg, developing in little water, are considerably retarded.

Date	Time in days	Egg with capsule in the stage of the yolk plug in small container with little water	Egg with capsule in the stage of the yolk plug in large container with a large quantity of water
February 23	0	Stage of the yolk plug	Stage of the yolk plug
March 13	18	Embryo in the stage of the tail bud; short gill primordia; large, sligthly pigmented yolk sac	Embryo in the stage of the tail bud; long gill primordia small yolk sac showing abundant pigmentation.
March 14	19	Very small eyes; no band-like pigment zone in fin fringe	Large eyes; commencement of formation of band-like pigment zone in fin fringe
March 17	22	Very small eyes; no pigmentation of fin fringe; hardly any yellow pigment cells; embryo has not yet hatched from the capsule	Very large eyes; band-like pigment zone in fin fringe; yellow pigment cells; embryo has not yet hatched from the capsule
March 18	23	Very small eyes; no pigmentation of fin fringe; embryo has not yet hatched from the capsule	Very large eyes; abundant pigmentation; yellow pigment cells; head-tail length 14.2 mm; gills with well-developed ramifications; early embryonal stage

with a large quantity of water. The development of eggs in a large quantity of water is always more rapid than that of eggs in little water. Whereas table 1 does not contain any data as regards hatching of embryos, table 2 teaches us that hatching occurs considerably more rapidly when the egg is reared in a large quantity of water than when it is reared in little water.

Fig. 3, finally, shows that the head-tail length of the embryos from eggs with intact capsules reared in little water is considerably smaller than that of corresponding eggs reared in a large quantity of water. In the first experiment the head-tail lengths after 23 days were 11.8 and 14.8 mm, respectively; in the other experiment (selected at random) the lengths after 25 days amounted to 12.6 and 15.3 mm, respectively.

CONCLUSION: In eggs developing in little water the growth and differentiation are considerably retarded.

The experiments represented in table 3 and in figs. 4 and 5 give some idea of the experiments carried out with eggs in the neurula stage. In these experiments the development of eggs with and without capsules in Holtfreter's solution was compared. Also in this case it appeared that the development of decapsuled eggs proceeds more rapidly than that of eggs with intact capsules. The criteria for the degree of development

FIG. 3.Diagram of two inhibition experiments with *Ambysto-ma* eggs. The eggs were placed in large containers and in small ones and had intact capsules (see fig. 2). Abscissa : time in days after the beginning of the experiment. Ordinate : head-tail length of the embryos in mm. High values : egg in a large quantity of water. Low values : egg in little water. In both experiments the embryo developing a large quantity of water appeared to attain a greater lenght than the embryo that had only little water at its disponal.

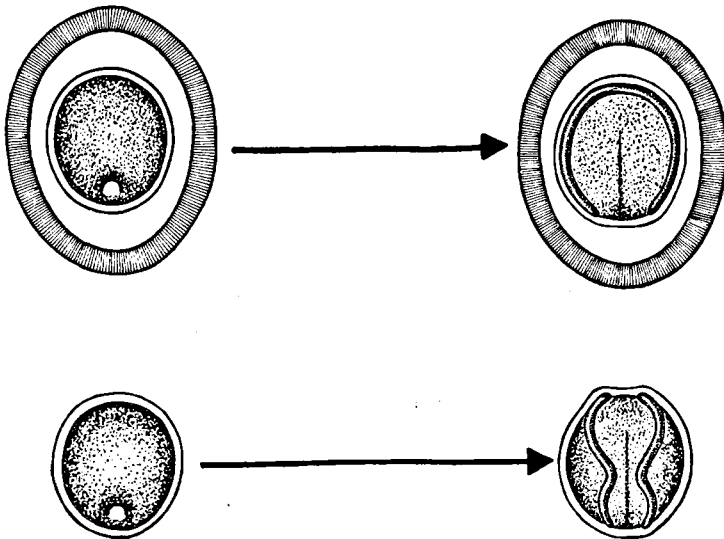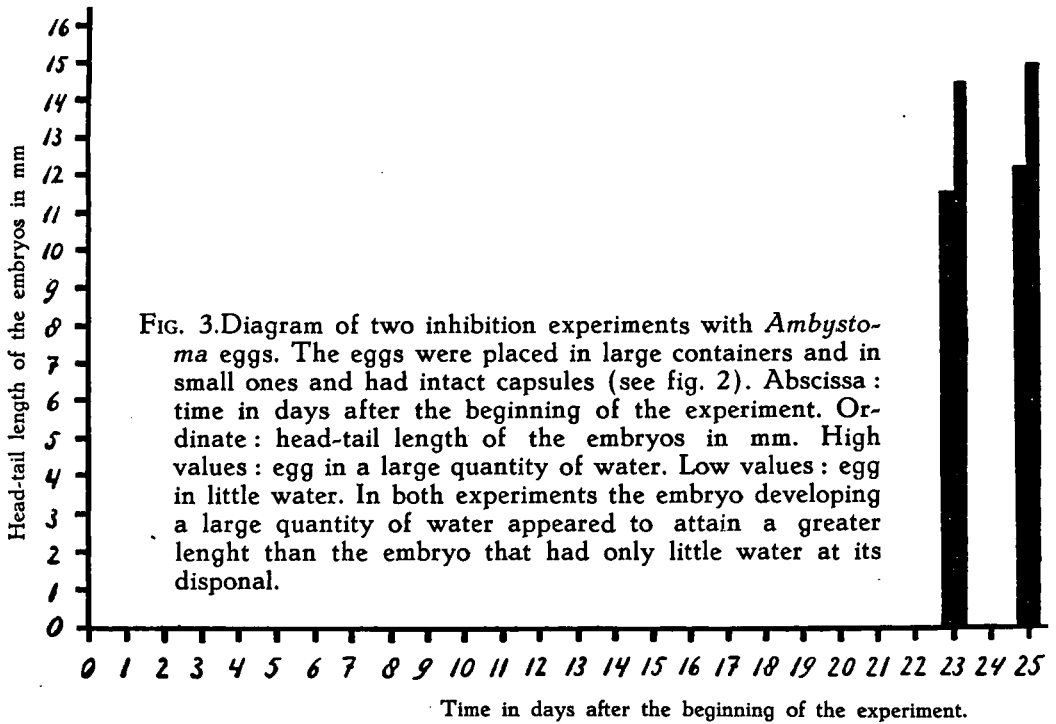Time in days after the beginning of the experiment.

FIG. 4. Inhibition experiment with *Ambystoma* eggs in the stage of the yolk plug. The medium was Holtfreter's solution.
A : after 56 hrs. the egg with intact capsule has only reached the stage of the young neurula.
B : after 56 hrs. the egg without capsule has attained the stage of the old neurula.

TABLE 3. Inhibition experiment with *Ambystoma* eggs in the neurula stage. The medium was Holtfreter's solution. The capsule of the one egg was removed, that of the other egg was left intact (see fig. 5). The growth and differentiation of the egg with intact capsule are considerably retarded.

Date	Time in hrs.	Egg with capsule in the neurula stage	Egg without capsule in the neurula stage
March 12 19.30 h.	0	Neurula stage	Neurula stage
March 13 18.30 h.	23	Neural folds still far apart	Neural folds touch each other
March 13 22 h.	$26^1/_2$	Neural folds touch each other, but are not yet grown together.	Middle portions of neural folds grown together along a considerable stretch
March 13 23 h.	$27^1/_2$	Neural folds not yet grown together	Neural folds grown together except the foremost parts
March 14 19 h.	$47^1/_2$	Line of separation between neural folds still visible	Neural folds completely grown together

TABLE 4. Inhibition experiment with *Ambystoma* eggs in the stage of the tail bud. The medium was Holtfreter's solution. The capsule of the one egg was removed, that of the other egg was left intact (see fig. 6). The growth and differentiation of the egg with intact capsule are considerably retarded.

Date	Time in days	Egg with capsule in the stage of the tail bud	Egg without capsule in the stage of the tail bud
March 19	0	Stage of the tail bud	Stage of the tail bud
March 21	2	Eye spot not yet visible	Eye visible as a dark spot
March 22	3	Eye spot not yet visible	Eye visible as a black spot
March 24	5	Small eyes (295 x 213 μ); few melanophores; few yellow pigment cells in head and fin fringe; head-tail length 9.3 mm	Large eyes (410 x 377 μ); many melanophores; many yellow pigment cells in head and fin fringe; yellow spots arranged in a band; head-tail length 11.1 mm

were in this case the formation and the closing of the neural folds.

CONCLUSION : In eggs with intact capsules the formation and closing of the neural folds are considerably retarded.

Finally, some experiments with eggs in the stage of the tail bud will be discussed (tables 4 and 5, figures 6 and 7). Here too the development of eggs with and without capsules in Holtfreter's medium was compared. The most rapid development, for all criteria was found in decapsuled eggs.

TABLE 5. Inhibition experiment with *Ambystoma eggs* in the stage of the tail bud. The medium was Holtfreter's solution. The capsule of the one egg was removed, that of the other egg was left intact. The growth and differentiation of the egg with intact capsule are considerably retarded.

| Date	Time in days	Egg without capsule in the stage of the tail bud	Egg with capsule in the stage of the tail bud
March 7	0	Stage of tail bud	Stage of tail bud
March 12	5	Short gill primordia; large. slightly pigmented yolk sac	Long gill primordia; yolk sac almost disappeared; already some pigmentation of the belly
March 14	7	No pigment zone of fin fringe	Pigment zone of fin fringe
March 17	10	Slight pigmentation of fin fringe; few yellow pigment cells; slight pigmentation of belly	Pigment zone of fin fringe distinct; many yellow pigment cells; strong pigmentation of belly
March 19	12	Short gills with few ramifications	Long gills with strong ramifications
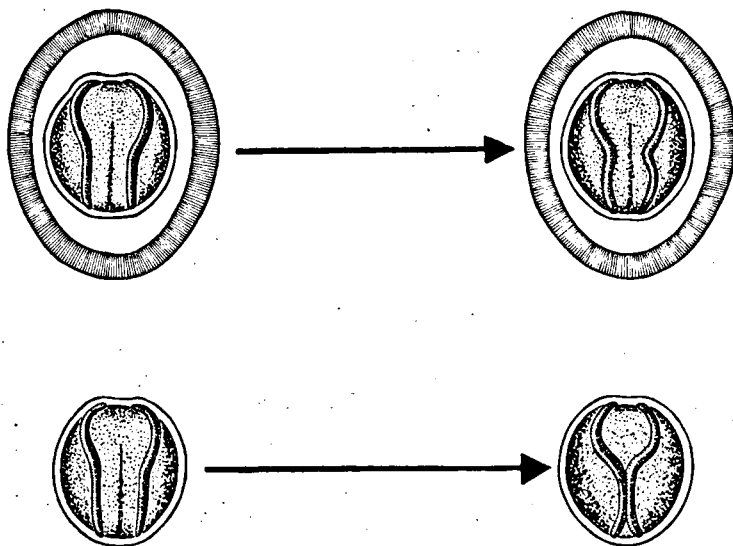
FIG. 5. Inhibition experiment with *Ambystoma* eggs in the neurula stage. The medium was Holtfreter's solution.

A : after 23 hrs. the egg with intact capsule shows only little progress in development.

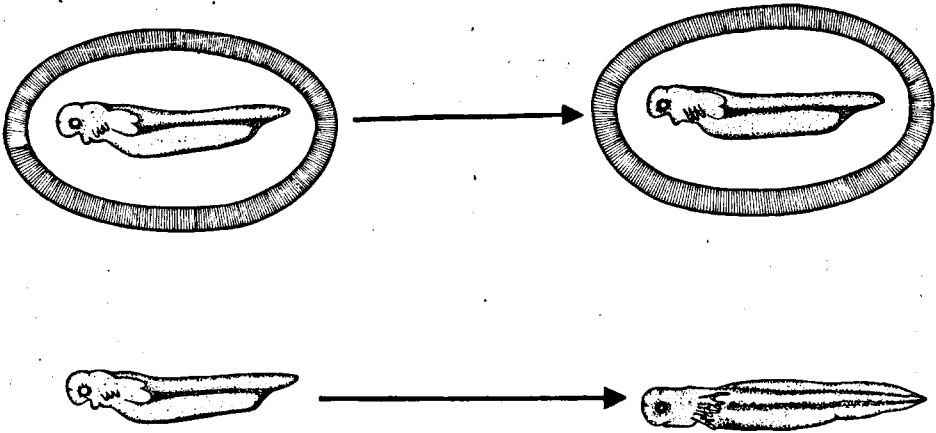B : after 23 hrs. the egg without capsule already shows the closing of the neural folds.

FIG. 6. Inhibition experiment with *Ambystoma* eggs in the stage of the
tail bud. The medium was Holtfreter's solution.
A : an egg with capsule is, after 117 hrs., still in the stage of the
tail bud. The gill primordia are slightly longer. B : an egg without
capsule has attained in 117 hrs. the first embryonal stage : eyes with
pigmentation, gill primordia with . ramifications, well-developed fin
fringe.

Fig. 7 shows that the head-tail length of the embryos reared from eggs
with capsules is considerably smaller than that of the embryos reared
from decapsuled eggs. In the first experiment the head-tail length after
5 days amounted to 9.3 and 11.1 mm, respectively. In the second experi-
ment 3 categories were compared : egg with capsule in little water, egg
with capsule in a large quantity of water and egg without capsule in a
large quantity of water. The results of the measurements were as follows :

egg with capsule in little water :   11.7 mm.
egg with capsule in a large quantity of water :   14.2 mm.
egg without capsule in a large quantity of water :   15.5 mm.
egg with a capsule in a large quantity of water :   14.2 mm.

The speed of development of the combination capsule-much water is
intermediate between the combinations capsule-little water and no cap-
sule-much water. This result is satisfactorily explained in terms of an
inhibiting substance excreted by the egg. The fact that in both experi-
ments with eggs having intact capsules in a large quantity of water the
same head-tail length was obtained demonstrates the accuracy of the
method.

CONCLUSION : The development of the embryo in an egg with intact
capsule is considerably retarded.

4. DISCUSSION OF EXPERIMENTAL RESULTS.

· On the strength of these experiments we can attribute a certain
growth-retarding activity to the presence of the capsule around the *Am-
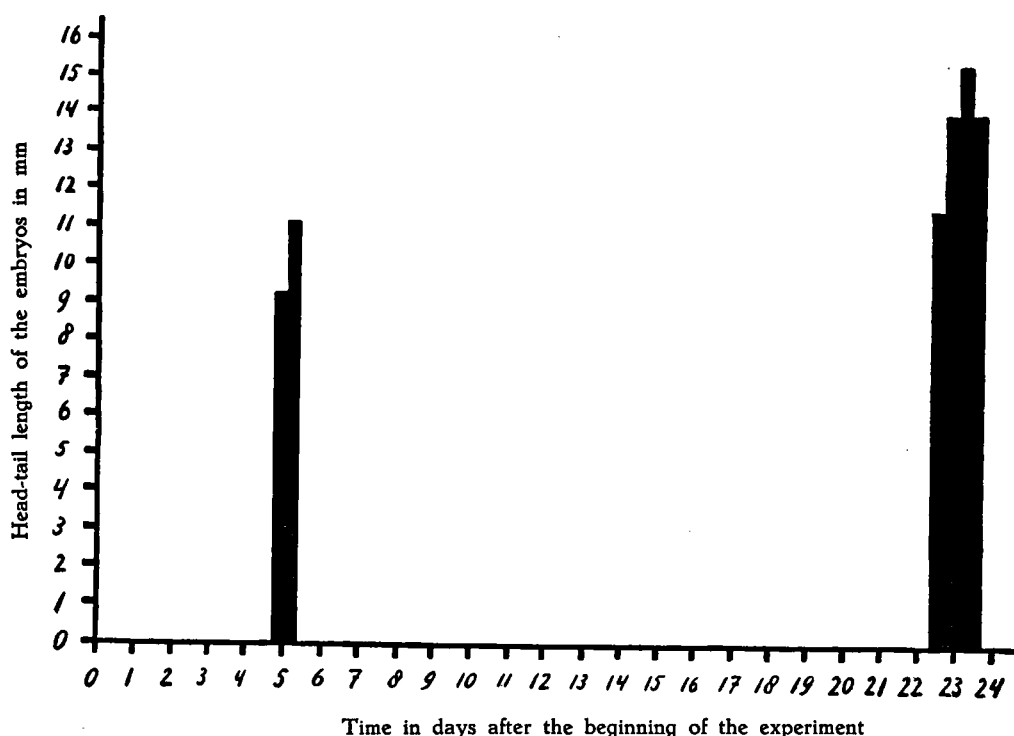bystoma* egg. The capsule acts as a retarding factor.

FIG. 7. Diagram of two inhibition experiments with *Ambystoma* eggs.
Of the eggs in the stage of the tail bud (after 5 days) the embryos
attained a head-tail length of 9.3 mm (egg with capsule) and 11.1
mm (egg without capsule), respectively. Of the eggs in the stage of
the yolk plug (after 23 days) the embryos attained a head-tail length
of 11.7 mm (egg with capsule in little water), 14.2 mm (egg with a
capsule in a large quantity of water), 15.5 mm (egg without a cap-
sule in a large quantity of water) and 14.2 mm (egg with a capsule in
a large quantity of water), respectively.

We must try to ascertain what may be the cause of this retardation.
Theoretically speaking, the following possibilities can be distinguished:
1. oxygen deficiency in eggs with intact capsules and in embryos develo-
   ping inside intact capsules ;
2. mechanical impediment by the capsule ;
3. an inhibiting substance excreted either by the capsule or by the egg
   into the capsule liquid ;
4. accumulation of carbon dioxide or other products of metabolism ;
5. lack of bodily exercise of the embryos.
   These possibilities will be briefly discussed.
AD. 1. A shortage of oxygen inside the capsules is quite likely at first
   sight, but must be excluded after a closer examination. It has been
   shown that oxygen diffuses freely through the capsule, so that the
   oxygen concentration required for the development of the egg or the
   embryo will therefore be reached within the capsule.

AD 2. Also the mechanical hindrance by the capsule can be practically left out of consideration, as the capsule does not enclose the egg tightly and is very soft in the later stages. The capsule therefore cannot be a hindrance to the development of the egg or the embryo.

AD 3 AND 4. The accumulation of an inhibiting substance and of carbon dioxide in the capsule can be discussed simultaneously.

Consequently, the following possibilities remain :

1. The factor of accumulation of a certain substance causing the retarding action (ad 3 and 4);

2. The possibility of lack of bodily exercise obtained by GOETSCH might plead in favour of the lack of bodily exercise (ad. 5). This author discovered some time ago that tadpoles grow more rapidly in a large quantity of water than they do in a small quantity (Cf. fig. 7, which is borrowed from GOETSCH's publication). The little tadpole (A) was reared in a small container with little water; the medium-sized one (B) in a container of the same size but in a large quantity of water, whereas the large tadpole (C) had as much water at its disposal as B had but was reared in a much larger container.
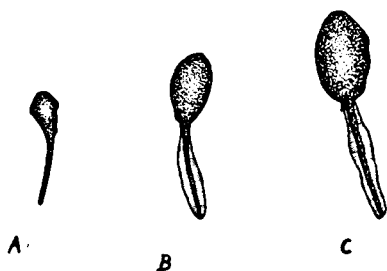

FIG. 8. Growth of tadpoles under different conditions.
    A : The little tadpole was reared in a small container with little water.
    B :The medium-sized tadpole was reared in a container of the same size but in a large quantity of water. C : The large tadpole was reared in a much larger container and had as much water at its disposal as B (after GOETSCH).

These experiments can, however, also be satisfactorily explained in terms of an inhibiting substance. Moreover, a considerable growth retardation of eggs with intact capsules was already observed in our experiments with eggs in the neurula stage, although there is no active muscle contraction in this stage of development, so that the factor of movement cannot be considered to be the cause of the differences in growth. Accordingly, only the possibility of an accumulation of the inhibiting substance remains.

A strong argument in favour of the inhibiting substance is to be found in the experiments in which the speed of development of the combination capsule-much water was intermediate between the combinations capsule-little water and no capsule-much water. The results obtained by DALCQ, FRÖSCHEL and BRANDES (1942), who, as was already mentioned in the introduction, were able to demonstrate a retarding effect of an aequous extract of *Viscum album* on the development of the eggs of *Rana fusca*, point in the same direction.

By assuming an inhibiting substance excreted by the eggs into the cap-sule liquid and retarding both the growth and the differentiation of the developing egg, the data obtained from the experiments can be satisfacto-rily explained.

5. THEORETICAL CONSIDERATIONS REGARDING THE POSSIBLE RELATION-SHIPS BETWEEN INHIBITING SUBSTANCES AND THE INCIDENCE OF MA-LIGNANT NEOPLASMS (CASU QUO MELANOSARCOMAS).

Melanosarcomas are of rather frequent occurrence in poikilothermous Vertebrates, in Fishes as well as in Amphibians and Reptiles. A concise survey, which is not quite exhaustive, is given here :

In Fishes, the melanotic tumours have chiefly been found in the hy-brids of the viviparous Cyprinodonts *Xiphophorus helleri* HECKEL and *Platypoecilus maculatus* GÜNTHER. Publications on the subject are those by HÄUSSLER (1928, 1934), KOSSWIG (1929a, 1929b, 1931), BREIDER (1939a, 1939b), GORDON (1936a, 1936b, 1937, 1941, 1946, 1948, 1951), REED and GORDON (1931), GORDON and FLATMAN (1943), GORDON and SMITH (1938a, 1938b), GRAND, GORDON and CAMERON (1941), GOR-DON and LANSING (1943).

The author has found a carcinoma in 11 specimens of the black variety of *Xiphophorus helleri* HECKEL, originating from the epidermis of the base of the tail and infiltrating into the corium, the subcutaneous connec-tive tissue and the musculature. One gets the impression that the tumour cells, the nuclei of which show a strongly polymorphous character, dis-perse the melanophores and afterwards overgrow them. The tumour process is attended bij a great increase in the number of melanophores. Probably the outgrowth of the melanophores is primary ; it is secon-darily followed by the outgrowth of the epidermis cells. For further details the reader is referred to the publications by the author on the sub-ject (STOLK, 1950b, 1951, 1953b). Just as in melanosarcomas the melano-phores play an important part in the formation of skin carcinomas.

Also in Amphibians some melanomas are known. KRONTOVSKY (1916) for instance found a subcutaneous melanoma in the axolotl *Siredon mexi-canum* (*Ambystoma mexicanum*), while SHEREMETIEVA-BRUNST and BRUNST (1948) discovered various cases of epidermal melanomas, also in the axolotl *Siredon mexicanum* (*Ambystoma mexicanum*). SHEREME-TIEVA BRUNST and BRUNST were able to obtain a number of specimens with melanomatic tumours by crossing a male specimen with a female one (both of the grey variety) which both showed small melanomas. This melanoma can therefore be considered to be a neoplasm which has developed on a genetical basis.

Finally, the melanomas of Reptiles. BALL (1946) described two cases of melanoma in the pine snake (*Pituophis melanoleucus*). A female spe-cimen possessed a skin tumour of the tail, a male specimen showed one in the labial area. Skin melanomas have also been reported in the reti-culated python (*Python reticulatus*) by SCHLUMBERGER and LUCKÉ and by LUCKÉ and BREEDIS (see SCHLUMBERGER and LUCKÉ, 1948).

The author has found a number of irregularly scattered nodules about 0.5 cm in diameter on the skin of seven specimens of the Lacertid *Lacerta agilis* L. (STOLK, 1950b, 1951, 1953c). On histological examination these

nodules showed a strong hyperkeratosis, with, in some places, a change into carcinoma planocellulare. The excessive formation of horn and the characteristic epithelium pearls, known from human pathology, were also present in these tumours. The lizards were practically exclusively fed on mealworms and were exposed to diffuse daylight, instead of direct sunlight which these animals need. As the carcinoma planocellulare is known to be, at least partially, one of the so-called irritation cancers, it is probable that external circumstances, in this case the small amount of light, have played a certain part in the genesis of these carcinomas. These tumours occurred in all seven animals, in which no other special factors are known that might have contributed to the stimulation of this carcinoma. Animals that were exposed to direct sunlight and also fed on mealworms, were not affected. Also in these skin carcinomas of *Lacerta* a considerable increase in the number of melanophores was observed. For further details the reader is again referred to STOLK (1950b, 1951, 1953c).

In the skin carcinoma of *Lacerta agilis* L., as well as in that of *Xiphophorus helleri* HECKEL (black variety) the number of melanophores is greatly increased. For the purpose of analysing the genesis of the melanophores more closely in relation to these carcinomas, a part of the caudal fin was removed in a good number of specimens of the "Hamburger hybrid" of *Xiphophorus* (head-tail length : 4—5 cm). This hybrid, in which the cranial part of the body is red and the caudal part black, was obtained by interbreeding the red variety of *Xiphophorus helleri* HECKEL and the cultivated form Black Molly. The animals invariably stood the operation well. Some time after the elimination of the caudal fin, varying from 5 tot 8 days, a colourless fin fringe developed from the black tail stump. This fringe in which the rays are quite distinct, grew continuously larger and remained perfectly colourless in the beginning, but later black rays of pigment occurred, starting from the black stump, the colourless tail fin gradually resuming the black appearance.

. Histological examination revealed that the melanophores do not appear simultaneously in the whole of the regenerated portion, but at first only in the most proximal areas and only afterwards in the distal parts. In this way the normal distribution of the melanophores in the fin is ultimately restored.

One might suppose that the melanophores of the tail stump start dividing, but since adult cells do not divide as a rule and, moreover, isolated melanophores are found here and there, we have to assume that the multipotent cells of the connective tissue present in the regenerated area have been formed into melanophores. The evident explanation of this process is, in our opinion, that a certain substance diffuses from the tail stump into the regenerated part and induces the differentiation of the multipotent fibroblasts of the connective tissue.

It has been mentioned that both the melanosarcomas and the skin carcinomas of *Lacerta* and *Xiphophorus* are accompanied with a considerable increase of the number of melanophores situated in the corium. In addition, we have pointed to the possibility of a material cause of the multiplication of the pigment cells, viz. the action of a hypothetical activating substance transforming multipotent fibroblasts into melanophores, the arguments for this assumption having been derived from the study of the regeneration of the tail in the Hamburger hybrid of *Xiphophorus*.

This point of view can also be applied to our discussion regarding the incidence of melanosarcomas, for – if we follow this train of thought – the melanosarcomas can be considered to be an extreme case of multiplication of pigment cells, without the epithelial outgrowth of the secundarily developing carcinoma subsequently overgrowing the mass of pigment cells. In the skin carcinoma the epithelial outgrowth may have prevented the primary outgrowth of the melanophores from developing into a melanosarcoma. The primary growth process is completely inhibited by the secundar one.

If we attempt to explain the origin of melanosarcomas, our discussion must be focussed on the remarkable, even more or less paradoxally striking phenomenon that these tumours are mainly found among pigment-deficient individuals. Some relevant data are given here, but the author is well aware of the fact that this survey is far from complete.

BOYD (1947) and MAC CALLUM reported that melanosarcomas are of frequent occurrence among white and grey horses, even to such an extent that every white or grey horse which attains a great age has a fair chance of dying from melanosarcoma.

Also in man a similar relation is found. It appears from the available statistical data that these tumours show a pronounced preference for the fair types. As the data from different countries do not completely correspond, an exact figure cannot be given and only the results obtained by MACCALLUM are cited here. This author indicated that on a total of 12000 autopsies melanoma was found in ten cases only. In spite of the fact that a fair percentage of negros occurred in this material, the bearers of these tumours invariably belonged to the white race.

The author was able to demonstrate a similar phenomenon in the black variety of *Xiphophorus helleri* (STOLK, 1950b). It has been previously mentioned that tumours in these black swordtails neoplasms are frequently found which usually occur in the area of transition between trunk and tail and on histological examination show the characteristics of melanosarcoma.

At first sight it seems strange that this neoplasm, occurring in a fish rich in pigment and obtained by repeated crossing, is mentioned in connection with the melanosarcomas which occur in pigment-deficient individuals. If, however, the internal organs of the black swordtail are examined, they appear to be distinctly deficient in pigment in contradistinction to the corium which shows abundant pigmentation, so that our classification proves to be justified, at least to a certain extent. The internal organs are mostly very pale in colour, much lighter than in the silvery or blue swordtail. The liver for instance is yellow instead of dark brown, the digestive tract is white instead of pink, the gonad white instead of ochre. In addition, the peritoneum shows hardly any pigmentation at all.

The classification of the black *Xiphophorus* among the pigment-deficient individuals is therefore a less artificial one than it seems at first sight. Moreover, local accumulations of pigment in the skin are by no means rare in white and grey horses (grey spots in the so-called dapple-greys). A similar local pigmentation apparently develops in the black swordtail in an excessive way.

Resuming, it can be concluded that melanosarcomas show preference for pigment-deficient individuals or, properly speaking, for those pig-

ment-deficient individuals in which a local accumulation of pigment may occur.

Can a satisfactory explanation of the combination melanosarcoma/ pigment-deficient individual be given? The phenomenon that a certain organism possesses a certain amount of melanophores which, after a period of normal activity, multiply excessively and form a malignant neoplasm, can, in our opinion, indeed be satisfactorily explained, or at least be made highly probable, by assuming that, exactly as during the development of the *Ambystoma* egg, an inhibiting substance is involved.

It has been mentioned in the introduction that the author (STOLK, 1953a) was able to distinguish three phases in the inhibiting process of *Allium Cepa* roots, viz.,

a. a phase of inhibition in which the inhibiting substance gradually decreases the speed of growth of the roots ;

b. a phase of equilibrium in which the inhibiting substance is not capable of reducing the speed of growth any further, so that the speed of growth remains practically constant ;

c. a phase of recovery in which the roots show an increased speed of growth again.

A process of inactivation of some inhibiting substance might well be the fundamental cause of the incidence of malignant neoplasms. This phenomenon would - at least to a certain extent - be comparable with phase *c* of the above-mentioned classification, normal growth, on the other hand, with phase *b*.

It is feasible that the "melanophore-inhibitor" must be present in the tissue-fluids in a certain concentration, thus preventing the abnormal multiplication of the melanophores.

This concentration cannot be an invariable one, because at a certain specific age of the individual neoplasmatic growth occur in a number of cases. Considered from the point of view of inhibition this is made possible by an inactivation of the inhibiting substance, or, what is more likely, by the fact that the inactivation exceeds the production of the inhibiting substance. The concentration falls off and is ultimately inadequate ; the multipotent cells of the connective tissue lack their natural inhibiting factor and consequently start forming an excess of melanophores, which in the end wreck the organism by their toxic products of metabolism.

This process is diagrammatically represented in fig. 9. Although it makes it probable that melanosarcoma occurs at a higher age, it does not explain the preference of this type of neoplasm to the pigment-deficient individual. For an explanation figs. 9 and 10 should be compared. As is indicated in fig. 10, the individual which is rich in pigment possesses a great number of pigment cells and consequently a greater potency of transforming multipotent fibroblasts into pigment cells. This means that, in the normal condition, the concentration of the melanophore inhibitor must be considerably higher in an individual rich in pigment, than in a pigment-deficient one and consequently the line of the inhibiting substance is at a higher level in the diagram (fig. 10) as compared with fig. 9, even to such an extent that during the inactivation the concentration remains above the minimum value as a rule. The result is that the formation of a tumour is prevented.

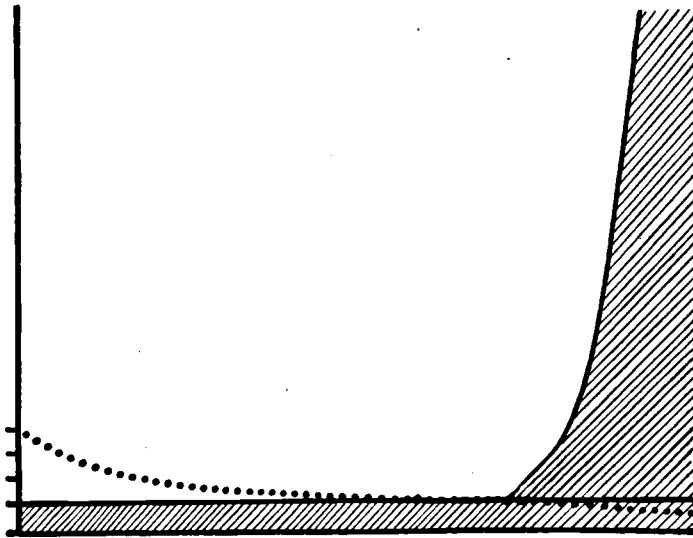By assuming the existence of an inhibiting substance that is subjected

FIG. 9. Relation between the concentration of an inhibiting substance and the number of melanophores in an individual poor in pigment. Possibility of the development of melanosarcoma. Abscissa: age; ordinate: number of melanophores; dotted line: concentration of the inhibiting substance.
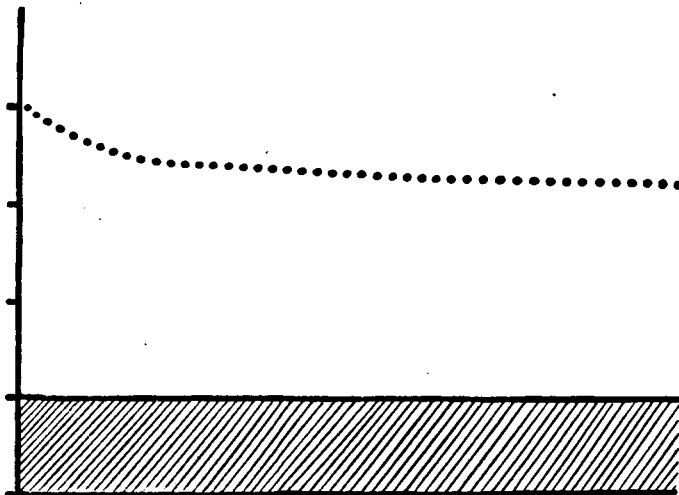

FIG. 10. Relation between the concentration of an inhibiting substance and the number of melanophores in an individual rich in pigment. No possibility of development of melanosarcoma. Abscissa: age; ordinate: number of melanophores; dotted line: concentration of the inhibiting substance.

to some inactivation process just as inhibiting substances of plants, several facts can be satisfactorily explained. The author has also attempted to make it possible that in the development of melanosarcoma and the skin carcinoma of *Lacerta* and *Xiphophorus* a melanophore substance is involved. However, is it possible to relate the melanophore activator and the melanophore inhibitor ?

It should be born in mind that a growth phenomenon can, in the first place, be considered from a "positive" point of view, i.e., as a causal result of the action of some growth substance. In the second place, however, it can be considered from a "negative" one, i.e., as a causal result of the inactivation of an inhibiting substance. If we apply an analogous interpretation in our case, the melanophore growth factor diffusing from the tail stump into the regenerated caudal fin would inactivate the melanophore-inhibiting substance present in the regenerated part, with the result that multipotent fibroblasts would be transformed into melanophores. In this way the two substances could be linked.

Does the literature on the subject provide any data which are possible points of contact with this inhibition theory of the origin of melanosarcomas ? GORDON et al. (1931, 1936a, 1936b, 1937, 1938, 1941, 1943, 1946, 1948, 1951) who, as was mentioned before, described a number of melanomas in hybrids of *Xiphophorus helleri* and *Platypoecilus maculatus*, attempted to give an explanation of the etiology of these tumours. These workers took the occurrence of the so-called macro- and micromelanophores as a starting point. For a short survey of their conception a quotation from the pertaining recapitulation by SCHLUMBERGER and LUCKÉ (1948) is given here (p. 684):

> "Swordtails *Xiphophorus helleri* possess only micromelanophores, or no melanophores at all ; platyfish *Platypoecilus maculatus* bear both micro- and macromelanophores. It is the platyfish macromelanophore factor introduced into the hybrid that produces melanosis and melanotic neoplasms. Although the dominant inherited factor responsible for melanosis is that for macromelanophores of the platyfish, alone it is ineffectual. The swordtail strain also plays its part in the production of the lesion, since it contributes hereditary factors that initiate the multiplication of the macromelanophores in the hybrids."

The melanosis, which is considered by GORDON et al. and by the author to be the preceding stage to the melanotic neoplasms, would initiate by the introduction of the macromelanophore factor of *Platypoecilus* into the hybrid *Xiphophorus* x *Platypoecilus*. When seen in the light of the inhibition theory, this factor lacks its anti-factor (the inhibiting substance) which it did possess in *Platypoecilus*. An increase in the number of melanophores, leading to melanosis and subsequent tumour formation, would be the result.

The remarkable phenomenon that melanosarcomas occur mainly in pigment-deficient individuals has been tentatively explained by the author by means of a hypothetical melanophore inhibitor which is similar to the inhibiting substance active in the development of the *Ambystoma* egg. The inhibiting substance present probably shows some quantitative relation to the number of melanophores and would prevent the development of melanosarcoma. If, however, this concentration falls off below the

minimum value, which occurs more easily in pigment-deficient individuals than in individuals rich in pigment, the etiological moment for the incidense of melanosarcoma would be given.

6. SUMMARY OF RESULTS.

1. Eggs of *Ambystoma mexicanum* with intact capsules show in a small container with little water a slower speed of growth and a slower differentiation than in a large container with a large quantity of water.
2. Eggs of *Ambystoma mexicanum* with intact capsules show in Holtfreter's solution a slower speed of growth and a slower differentiation than corresponding eggs, also reared in Holtfreter's solution, after removal of the capsules.
3. Most probably the retardation of the process of growth and differentiation of *Ambystoma* eggs with intact capsules is due to an inhibiting substance excreted by the egg into the capsule liquid.
4. This self-inhibition of the *Ambystoma* egg corresponds to a certain extent to the auto-inhibition of seeds of *Trifolium* and *Beta* (FRÖSCHEL, 1939, 1940), to the auto-inhibition of roots of *Fuchsia* and *Pelargonium* (STOLK 1952) and to the auto-inhibition of roots of *Allium Cepa* (STOLK 1953a).
5. The possibility is mentioned that a similar inhibiting substance might play a part in the occurrence of malignant neoplasms, in casu of the melanosarcomas.

Literature cited

BALL, H. A. —
    1946 Melanosarcoma and rhabdomyoma in two pine snakes, Pituophis melanoleucus. Cancer Research *6*, 134—138.
BOYD, W. —
    1947 A textbook of pathology.
BREIDER, H. —
    1939a Über die Vorgänge der Kernvermehrung und -degeneration in sarkomatösen Makromelanophoren. Z. wiss. Zool. *152*, 89.
    1939b Über die Pigmentbildung in den Zellen von Sarkomen albinotischer und nichtalbinotischer Gattungsbastarde lebendgebärender Zahnkarpfen. Z. wiss. Zool. *152*, 107.
DALCQ, A., P. FRÖSCHEL et J. BRANDES —
    1942 Action d'une substance frénatrice d'origine végétale sur le développement de Rana fusca. Acta biologica belgica *4*, 409.
FRÖSCHEL, P. —
    1939 Onderzoekingen over de physiologie van de kieming. I Remstoffen. Natuurwetenschappelijk Tijdschrift *21*, 93—116.
    1940 Untersuchungen zur Physiologie der Keimung. Zweite Mitteilung. (Hemmstoffe, Fortsetzung). Biologisch Jaarboek Dodonaea *7*, 73—116.
GORDON, M. —
    1936a The production of spontaneous melanotic neoplasms with a single type of melanophore in hybrid fishes. Amer. Nat. *70*, 50.
    1936b The production of spontaneous melanotic neoplasms at birth in hybrid fishes. Amer. Nat. *70*, 51.
    1937 The production of spontaneous melanotic neoplasms in fishes by selective matings. Am. J. Cancer, *30*, 362.
    1941 Genetics of melanomas in fishes. V. The reappearance of ancestral micromelanophores in offspring of parents lacking these cells. Cancer Research *1*, 656.
    1946 Genetics of ocular-tumor development in fishes. J. Nat. Cancer Inst. *7*, 87.
    1948 Effects of five pigment genes on the site of melanomas in fishes and the influence of two color genes on their pigmentation. In "The Biology of Melanomas", Spec. Publ. New-York Acad. Sc. *4*, 216.

1951 The variable expressivity of a pigment cell gene from zero effect to melanotic tumor induction. Cancer Research *11*, 676.

GORDON, M. and F. FLATMAN —
1943 The genetics of melanoma in fishes. VI. Mendelian segregation of melanophore reaction types in embryos of a melanomatous mother. Zoologica *28*, 9.

GORDON, M. and W. LANSING —
1943 Cutaneous melanophore eruptions in young fishes during stages preceding melanotic tumor formation. J. Morph. *73*, 231.

GORDON, M. and G. M. SMITH —
1938a Progressive growth changes of a heritable melanotic neoplastic disease in fishes from the day of birth. Am. J. Cancer *34*, 255.

GORDON, M. and G. M. SMITH —
1938b The production of a melanotic neoplastic disease in fishes by selective matings. IV. Genetics of geographical species hybrids. Amer. J. Cancer *34*, 543.

GRAND, C. G., M. GORDON and G. CAMERON —
1941 Neoplasm studies. VIII. Cell types in tissue culture of fish melanotic tumours compared to mammalian melanomas. Cancer Research *1*, 660.

GROOT, B. DE and J. J. DUYVENÉ DE WIT —
1949 Copulin and ovipositor growth in the bitterling (Rhodeus amarus, Bl.). Acta endocrinologica *3*.

HÄUSSLER, G. —
1928 Über Melanombildungen bei Bastarden von Xiphophorus hellerii und Platypoecilus maculatus var. rubra. Klinische Wochenschrift 7, 1561.
1934 Über die Melanome der Xiphophorus-Platypoecilus bastarde. Zeitschrft für Krebsforschung *40*, 280.

JASKI, C. J. —
1939a Ein Oestruszyklus bei Lebistes reticulatus (Peters). Proc. Kon. Ned. Akad. v. Wetensch. *42*, 201.
1939b De elevatie van Lebistes reticulatus. Thesis, Utrecht.

KÖCKEMANN, A. —
1934 Ueber eine keimungshemmende Substanz in fleischigen Früchten (Vorläufige Mitteilung). Berichte der deutschen botanischen Gesellschaft *52*, 523.

KOSSWIG, C. —
1929a Melanotische Geschwülstbildungen bei Fischbastarden. Münch. med. Wochenschr. *76*, 1070. Zool. Anz. Suppl. *4*, 90.
1929b Zur Frage der Geschwülstbildungen bei Gattungsbastarden der Zahnkarpfen Xiphophorus und Platypoecilus. Zeitschr. f. indukt. Abstamm. u. Vererb. *52*, 114.
1931 Über Geschwülstbildungen bei Fischbastarden. Zeitschr. f. indukt. Abstamm. u. Vererb. *59*, 61.

KRONTOVSKY, A. —
1916 Comparative and experimental pathology of tumors (Russian). Kiev. (Cited by Sheremetieva-Brunst and Brunst, 1948).

LUCKÉ, B. and BREEDIS —
1948 Not published. Cited by Schlumberger and Lucké: Tumors of Fishes, Amphibians and Reptiles. Cancer Research *8*, 657—753.

MAC CALLUM, W. G. —
A textbook of pathology. Seventh edition. W. B. Saunders Company, Philadelphia and London.

REED, H. D. and M. GORDON —
1931 The morphology of melanotic overgrowths in hybrids of Mexican killifishes. Am. J. Cancer *15*, 1524—1537.

RUGE, U. —
1939 Zur Physiologie der genuinen keimungshemmenden und keimungsbeschleunigenden Stoffe von Helianthus annuus. Zeitschrift für Botanik *33*, 529—571.

SCHLUMBERGER, H. G. and B. LUCKÉ —
1948 Tumors of Fishes, Amphibians and Reptiles. Cancer Research *8*, 657—753.

SHEREMETIEVA-BRUNST, E. A. and V. V. BRUNST —
1948 Origin and transplantation of a melanotic tumor in the axolotl. In "The Biology of Melanomas", Spec. Pub. New-York Acad. Sc. *4*, 269—287.

STOLK, ANTH. —
1950a Histo-endocrinologische analyse van de graviditeitsverschijnselen bij de

Cyprinodontide Lebistes reticulatus. (With an English summary). Thesis, Utrecht.

1950b Enige gevallen van gezwellen en ontstekingen bij poikilotherme Vertebraten, een bijdrage tot de Vergelijkende Pathologie. (With an English summary). Thesis, Utrecht.

1951 Enige vergelijkend-pathologische aspecten van het kankervraagstuk. Eerste Jaarboek van de Vereeniging Het Nederlandsch Kankerinstituut *1*, 89—97.

1952 Growth-inhibiting substances in the roots of Fuchsia hybrida and of Pelargonium zonale. Beaufortia 17.

1953a The root-inhibiting substance of Allium Cepa. Beaufortia *28*.

1953b Tumours of fishes. III. Carcinoma of the epidermis in the black variety of the viviparous Cyprinodont Xiphophorus hellerii Heckel. Proceedings Koninkl. Nederl. Akademie van Wetenschappen, Amsterdam C *56*, 143—148.

1953c Hyperkeratosis and carcinoma planocellulare in the lizard Lacerta agilis L. (Preliminary note). Proceedings Koninkl. Nederl. Akademie van Wetenschappen, Amsterdam C *56*, 157—163.

WIESNER, J. —

1894 Pflanzenphysiologische Mitteilungen aus Buitenzorg IV. Vergleichende physiologische Studien ueber die Keimung europäischer und tropischer Arten von Viscum und Loranthus. Sitz. ber. d. Wien. Akad. d. Wiss., math.-naturw. Kl., Abt. I, *103*, 401.