Chemistry. — *Polyploidy in Frogs, induced by Colchicine.* By GÖSTA HÄGGQVIST.

(Communicated at the meeting of February 28, 1948.)

In 1937 BLAKESLEE published the first communication regarding polyploidy in plants, induced by treatment with certain chemical substances, including colchicine.

The genetic importance of the induced polyploidy is emphasized by BLAKESLEE in the following passage:

„Si l'induction du dédoublement du nombre de chromosomes par traitement chimique a une valeur générale, le cultivateur pourra travailler avec plus de précision pour diriger l'évolution des formes économiquement importantes, aussi bien celle des plantes propagées par des méthodes végétatives que des plantes multipliées à partir des graines. Il devrait être possible, par example, de synthétiser une race double-diploïde stable qui aurait la vigueur hybride et les caractéristiques désirées des tétraploïdes, à partir d'un hybride stérile. Le dédoublement du nombre des chromosomes donnerait à l'horticulteur des fleurs et des fruits plus grands; et pourrait être, par l'intermédiaire des triploïdes, l'origine d'une variété de types $2n + 1$."

„La tétraploïdie et la présence de chromosomes supplémentaires non balancés ont été des facteurs importants dans la constitution d'un grand nombre de nos meilleurs fruits et fleurs. En plus de l'augmentation des organes de la plante, la tétraploïdie a transformé une forme stérile en fertile, une plante dioïque en hermaphrodite, une plante annuelle en plante vivace, et a accru la résistance au froid. En Zoologie l'induction du dédoublement du nombre des chromosomes est un champ inexploré, mais qui sera probablement fécond. La possibilité d'induire expérimentalement le dédoublement du nombre des chromosomes est donc important pour la génétique pratique aussi bien que pour la génétique théorique."

This communication regarding the possibility of inducing polyploidy immediately gave rise to vigorous experimentation on the part of students of genetics and plant cultivators. They discovered that a number of other substances besides colchicine also had a similar effect. The first enthusiasm for the new possibilities that had thus been opened up, however, soon gave place to a more sober view. The polyploid forms were not always larger and more vigorous. It seems in fact, as pointed out by LEVAN (1942), that there is an optimal number of chromosomes for each kind of plant. Over and above that number the vitality of the plant decreases. Sometimes plants with the optimal number of chromosomes occur quite normally in nature. In such cases the introduction of artificial polyploidy can merely result in lowering the plant's vitality.

In other cases, however, where the existing forms have not acquired the optimal number of chromosomes, much may be gained by the induction of polyploid forms. Thus, in spite of all disappointments, experimental polyploidy has already proved to be a valuable aid in plant cultivation, and it is as yet too early to survey the further advances that are likely to be attained by this method.

As regards the induction of polyploidy in *animals*, certain progress has also been made. The most striking results in this respect have been gained not by chemical means, but by the application of a method with extreme temperatures — cold or heat —, which had long been employed by plant cultivators. In 1938 GRIFFITHS, by refrigerating newly fertilized eggs of *Triturus viridescens* down to 0°—3° C for 5 to 24 hours, succeeded in producing triploid larvae. In 1942 FANKHAUSER produced triploidy in *Triturus* by temperatures of 35°—37° C. The mortality was, however, very high. According to FANKHAUSER (1942), spontaneous polyploids are fairly commom in certain amphibia. In *Triturus viridescens* he had in 1942 found, among more than a thousand larvae, 26 heteroploid specimens. Spontaneous polyploidy has been observed also in other animals. It has most often been found in amphibia, at the earliest recorded date by G. and P. HERTWIG (1920) in *Rana esculenta*. According to SVÄRDSON (1945), the extant Swedish *Salmonidae*, with their high chromosome numbers, are probably natural polyploids of a now extinct stock.

As regards growth and size, FANKHAUSER could not observe any marked distinction between diploid and triploid animals, though the latter appeared to be more vigorous. The tetraploid larvae, on the other hand, were smaller and of slower growth; the pentaploid after a time developed edema and had little vitality.

The possibilities of inducing polyploidy in plants by means of colchicine naturally aroused expectations that this might be the case also in animals. It was, however, in animal cells that LITS (1934) first observed the peculiar effect of this alkaloid on the mitoses. BLAKESLEE (1938), for example, states that EMBODY and PHILLIPS had treated trout eggs, NOBLE and MATHEWSON frog eggs and aquarium fishes, with colchicine, though without effect, owing to the difficulty in getting the alkaloid to penetrate into the eggs. NEBEL (1937) as well as NEBEL and RUTTLE (1938) found that colchicine in concentrations stronger than 10^{-4} hindered the cell division after the first maturation division in eggs of *Arbacia punctulata*, if they were thus treated 10 minutes after the fertilization. The effect was similar to that in plant cells, in that the spindle was defective. PINCUS and WADDINGTON (1939) treated newly fertilized rabbit eggs with dilute solutions of alcohol, ether and colchicine. The concentration of the last-mentioned solutions was 0.00002—0.0005 %. They found that the colchicine especially impeded the development of the spindle, the cleavage of the cytoplasm and the movements of the pronuclei. In the same year KEPPEL and DAWSON inseminated frog eggs in accordance with the method of

RUGH. After a quarter of an hour the eggs were rinsed with water and after 15 minutes more they were separated without removing the gelatinous sheath. They were laid in colchicine solutions of varying concentration, ranging from 1 : 10000 to 1 : 1000000 and were kept in them continuously. The tests were made at 8° and 20° C, respectively. The first fissures were formed in solutions of 1 : 10000. In solutions of 1 : 1000000 the eggs on the second day varied from normal to those in which a "cap" of cells had been formed at the animal pole, but where the remainder of the egg was undivided. All the eggs — even those apparently normal —, were dead on the third day; in no case did the development go further than to an imperfect gastrulation. — MILLS (1939) studied the effect of colchicine on the mitoses of frogs and salamanders and found a retardation at metaphase, due to the non-development of the spindle. In the same year PAFF studied the effect of colchicine on 24 hours-old chick embryos. The result is not clear.

In 1940 WATERMAN subjected fish embryos, *Oryzias lapites*, in four different stages of development, to colchicine solutions of varying concentration, 10^{-5}—10^{-3}, for periods of varying length. He found the strongest concentration most "useful". He observed a general retardation and inhibition of cleavage and development, but also a number of abnormities in pigmentation, cyclops eyes and disorders in the shaping of the body — especially in the tail, which in certain cases was retarded or inhibited in development, etc. The periods during which the embryos were exposed to the action of the colchicine varied from 1 hour to 25 days. — In the same year HIGBEE injected 0.02 cm^3 of a 0.0001 % colchicine solution into hen's eggs, which had been brooded for 24 hours. She obtained in the adult stage one hen and two cocks with abnormally large combs and wattles; two tail-feathers of the cocks were abnormally long. The hen laid eggs after pairing with one of the cocks, but it is not known whether they were polyploid. DUNHAM and BANTA (1940) made experiments with eggs of *Daphnia longispina*, which were treated with colchicine in a concentration of 10^{-4}—10^{-8}, and obtained a number of abnormally developed individuals, but no form of polyploidy.

The attempts to induce polyploidy in animals by means of colchicine were thus not very encouraging. FANKHAUSER (1942) in fact states: "Everyone is aware of the great progress that has been made in the study of polyploidy in plants since the introduction of colchicine. With animals, this technique has not been very successful so far; we have to look for other methods for a controlled production of polyploids, which would be extremely desirable". As above mentioned, this author himself and his coworkers have attained interesting results with temperature shocks.

BÖÖK (1945) reports cold-shock experiments with eggs of *Triton taeniatus* and *cristatus*, by which he had obtained triploid larvae. He mentions that he had also made experiments with colchicine in varying concentrations, but without positive results, which he attributes to the fact that the egg-membrane is impermeable o colchicine. He considers it doubtful whether it is possible at all to induce polyploidy in animals with this alkaloid. He found no distinct difference in size between diploid and triploid animals. In order to determine the size of the nucleus, he measured the largest (2*a*) and the smallest (*b*) diameter and, after comparing the product ab^2 for diploid and triploid animals, found for different tissues a ratio of 1 : 1.5—1 : 1.74.

Own experiments.

Before proceeding to report my experiments, I may be permitted to mention certain circumstances which had unfavourably affected them and entailed serious interruptions in the investigations. In June my institute was to be removed from its former premises to a new building. In April and May the instruction was still being carried on in the former premises, but the preparations for the removal and the arangements for the fitting-up of the new ones almost completely hindered all scientific work on my part. In the new institute, building work was still going on. Here, however, in April the aquarium was in a sufficient stage of completion to enable me to use it, and in default of other scientific work, I was then tempted to induce polyploidy in animals by means of colchicine. As the building work was still proceeding, however, the aquarium could not be kept closed, and the distance between the old and the new institute prevented effective supervision.

For the purpose of these experiments, I selected the common Swedish frog, *Rana temporaria,* which pairs at that season. The literature seemed to me to indicate that I must use considerably weaker colchicine solutions than had been generally adopted in plant cultivation. It seemed to be also desirable to treat the fertilized oöcytes before any cleavages had occurred. When a multicellular stage has been reached, the colchicine may variously affect different cells, so that "mosaics" are produced.

As for the difficulty of enabling the colchicin solution to penetrate the gelatinous sheath protecting the frog egg, it occurred to me that it might be overcome in the following manner. When the eggs are laid, they sink to the bottom. Here the gelatinous sheath absorbs water and swells, whereupon the eggs with their sheaths float up to the surface. This process should make it possible to bring the colchicine into touch with the eggs. In fact, if the latter are laid in the colchicine solution the alkaloid should be absorbed together with the water and penetrate into the egg.

Investigations by JACOBIJ have shown that frogs are very insensitive to colchicine. They should not therefore be affected by the weak solutions that might be considered here. Nor could the sperms be expected to be unable to inseminate eggs in the weak solutions used, as they did not seem to affect the motility of the cells.

Proceeding from this reasoning, I placed 3 males and 3 females in each of six basins, where they immediately paired. When signs indicated that the time for egg-laying was approaching, I mixed solutions of colchicine in the different basins, so that they came to contain the alkaloid in the following concentrations: 1 : 50,000; 1 : 100,000; 1 : 200,000; 1 : 500.000; 1 : 1,000,000. I had also a basin without colchicine for purpose of control.

The frogs remained in the colchicinesolution for 48 hours, after which the alcaloid was washed away. The further development of the inseminated eggs thus occurred in pure water.

Observations.

Owing to an accident connected with the circumstances introductively recounted, the basin containing colchicine in the concentration of 1 : 500,000 was emptied, and I am therefore unable to state whether this concentration is serviceable or not. In the basins containing the concentrations 1 : 50,000, 1 : 100,000 and 1 : 200,000, respectively, the eggs died before any larvae had as yet developed. Whether any cleavages had occurred at all in these eggs, I am unable to state.

In the control basin eggs were laid and two days later also in the basin with colchicine in a solution of 1 : 1,000,000. In both cases larvae were developed. The control animals from the outset were somewhat larger than the colchicine animals, but the lead was soon made good.

When I returned from a journey to Finland which I made in the latter half of May, the control animals had died.

However, I had, in addition, a general basin for frogs, in which I kept animals for the general requirements of the institute. In this basin eggs had been laid earlier than in the experimental basins, at a date that had not been precisely recorded. On my return from Finland, the larvae that had been developed from these eggs (designated below by *x*) were considerably larger than the experimental animals. Those larvae now were used as controls.

The colchicine-larvae, however, grew more rapidly and, in course of time, became on an average considerably larger (Plate 1). Not all the colchicine animals, however, had grown with equal rapidity. Some of them developed at a normal rate. The latter also passed through their metamorphosis at the usual time or slightly later. In the Stockholm district this occurs towards the end of July or beginning of August. At the beginning of November the large larvae had not yet passed through any metamorphosis. Among these animals there are some that were completely devoid of any visible rudiments of extremities. In others the posterior extremities are well developed, whereas the anterior ones are completely lacking.

WATERMAN (1940), in his fish embryos treated with colchicine, had found malformations, especially in the tail region. In about one-third of the tadpoles similar anomalies were observed. They always affect the transition between the body and the tail. In some of the animals, one or more segments on the one side seem to be stunted in development, so that the tail, instead of continuing in the direction of the body-axis, makes a bend which may amount to about 45° or somewhat more. Usually, however, two bends are seen, first one at the base on one side, and then another more distally on the other side, so that a kind of bayonet-bend is produced. The tail then runs in the direction of the body-axis, but is displaced towards one side. No other anomaly in the development of the tail has been found. It is, on the contrary, longer and more powerful than in normal animals.

The tadpoles in which the tail forms an angle with the body-axis, when not disturbed, can swim in any direction. If, however, they are scared, they often move, owing to the oblique position of the tail, hurriedly in circles.

FANKHAUSER, in his polyploid salamander larvae, regularly found changes in their pigmentation. In those treated with colchicine most of them show a darker colour than the average in the normal larvae. Three of them had been albinos; one of them died, so that at present only two are left. They are faintly yellowish-white and semitransparent.

Some retardation of the development is usual in polyploid growths. It may be questioned, however, wether the complete absence of one or both pairs of extremities can be thus explained, or wether it may be presumed that the hereditary structure had been more profoundly affected.

It is also difficult to say how the curvature of the body-axis is to be explained.

I have not observed any other anomalies in the appearance of the animals.

Nuclear conditions. When the animals were at such early stages of development that chromosome counts could be successfully made, the former premises of the institution could not be used on account of the move, and the new premises were not yet in order. I afterwards tried to determine the chromosome number on entire epidermis cells from the tail-fins, but at metaphase the chromosomes lie so closely together that these attempts have hitherto failed. However, as FANKHAUSER has pointed out, the size of the nucleus is proportional to the chromosome number, haploid nuclei being smaller than diploid, the latter, again, smaller than triploid, and so forth.

Theoretically, the matter, as it seems to me, may be viewed as follows: — In amphibia the egg as a rule has not terminated its reduction division (meiosis) when it is inseminated. The colchicine should therefore check the meiosis. In that case the result should be a diploid female nucleus, which with the haploid sperm cell should produce a triploid individual. Should then the first division of the sperm-ovum likewise be checked, the result should be a hexaploid one. Nothing, however, indicates that such individuals have actually been produced.

If, on the other hand, the reduction division of the egg is already terminated when the insemination takes place, the colchicine would affect the first division of the sperm-ovum and a tetraploid individual should result.

In order to estimate the relative size of the nucleus I used a microphotography apparatus and projected the nuclei of the epidermis preparaions on paper with a magnification of 1000 ×, and outlined their contours. No selection was made, all the nuclei in the field of vision being outlined. In normal animals as well as in those treated with colchicine, the nuclei vary in form and size. The variations in the form of the nucleus are, however, similar in both. The size seems to show greater variations in the animals treated with colchicine. It cannot be said that in the latter the nuclei are always larger than in the former. But, as indicated in Fig. 1, it is evident that the colchicine-treated animals have considerably larger nuclei.

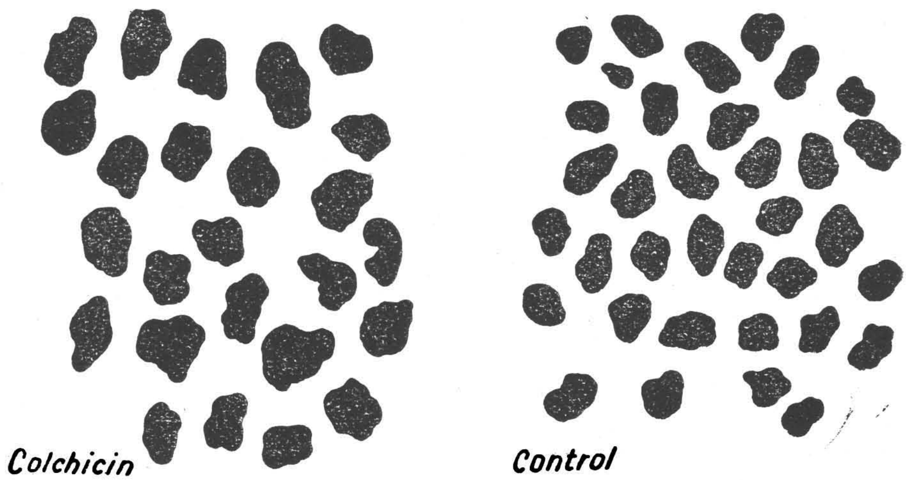

Fig. 1.

In order to obtain an idea of the ratio between the sizes of the nuclei, I sketched over five hundred epidermis cell-nuclei in a colchicine-treated and a control animal. In respect of each nucleus, I computed the longest diameter and the largest transverse diameter at right angles thereto. From the values thus obtained, I computed a mean diameter and, with the latter as a basis, estimated the nuclear volume regarded as a sphere.

As the nuclei are not actually spherical, but flattened and somewhat irregular, the nuclear volume thus estimated will obviously be larger than the real one. In both cases, however, the sources of error must be of the same relative magnitude, whence the figures obtained are comparable with one another.

For the control animals the estimated mean volume was $293.81 \pm 4.48\ \mu^3$, and for the colchicine-treated animals $548.87 \pm 17.28\ \mu^3$. The difference, $244 \pm 17.9\ \mu^3$, is statistically significant. The ratio is 1 : 1.87. This value lies between 1 : 1.5, a figure which might be expected in triploid animals, and 1 : 2.0, which might be expected in tetraploid ones; but considerably closer to the latter. As BÖÖK (1945) found rather marked variations in the nuclear ratios in different kinds of cells of *Triton taeniatus*, it may be presumed that the nuclear volume is not always directly proportional to the number of haploid chromosome sets. The degree of polyploidy is thus uncertain.

The following facts, however, seem to indicate that the colchicine-treated animals whose nuclear volume relatively to that of normal animals is in the ratio of 1 : 1.87 are tetraploid. Among the control animals designated with x, there were two that did not pass through the metamorphosis in normal time. By the 15th October only weak posterior extremities had been developed in those animals, but no anterior ones. In one of these animals, I measured 100 epidermis nuclei from the tail-fin and estimated the nuclear volume in the manner above described. The volume was $443.04 \pm 13.8\ \mu^3$. These nuclei were thus larger than in the normal control animals, but smaller than in the colchicine-treated ones. The difference relatively to the former was 149 ± 14.5 and relatively to the latter 105.83 ± 22.12. In both cases the difference is statistically significant. The nuclear volume relatively to that of the normal x-animals was 1 : 1.51. It may therefore be presumed that we are concerned here with spontaneous triploids. As the large colchicine-treated animals show still larger nuclei, clearly distinguishable from those of these presumed triploids, the former seem to me to probably tetraploid.

As above mentioned, among the colchicine-treated animals there were some small specimens that passed through the metamorphosis in normal time. They showed a nuclear volume which did not differ from that of the control animals. They are thus in all probability diploid. Nevertheless the colchicine treatment in certain cases had not been entirely ineffective. Some of them infact showed the same curvature of the tail that is otherwise found in many of the polyploid animals.

Summary.

1. Males and females of *Rana temporaria* were placed in basins. When signs indicated that the time for egg-laying approached, colchicine was mixed in the water so that the concentration in different basins ranged from 1 : 50.000 to 1 : 1.000.000. After 48 hours the colchicinesolution was washed away.
2. Larvae developed only in the concentration of 1 : 1.000.000.
3. Many of those larvae appear to be polyploid.
4. In *Rana temporaria* the triploid and tetraploid forms are giant-forms.
5. Colchicine induces not only polyploids but affects also in other ways the development of animals. Retardation of the metamorphose, changes of the pigment, disturbances of the bodily structure, checking of the development of special organs. These changes vary from individual to individual.

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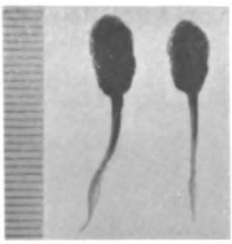

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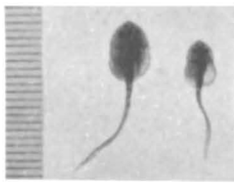

Colchicin

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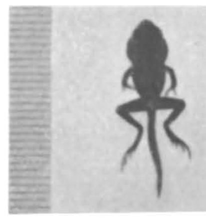

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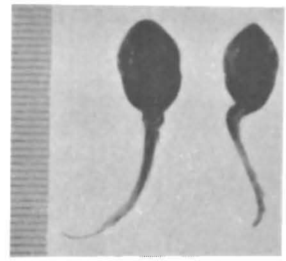

Colchicin

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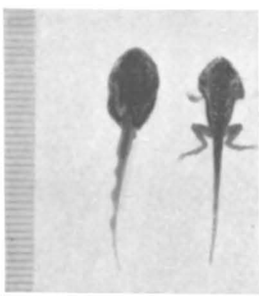

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Colchicin

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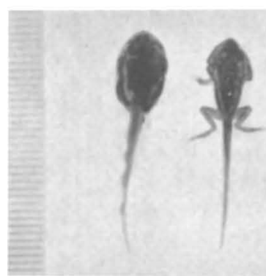

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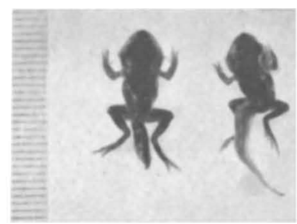

Colchicin
Diploides

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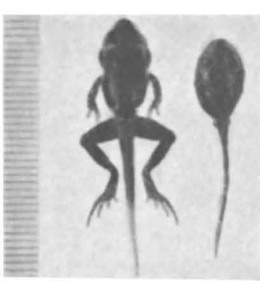

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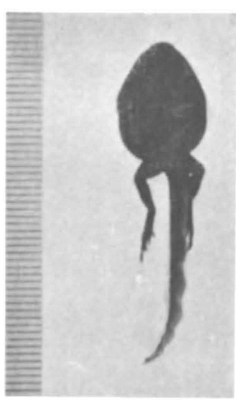

Colchicin

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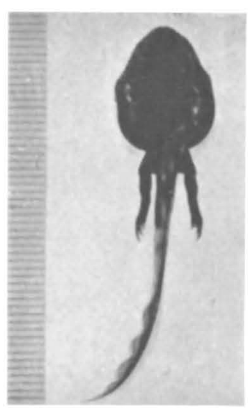

Colchicin

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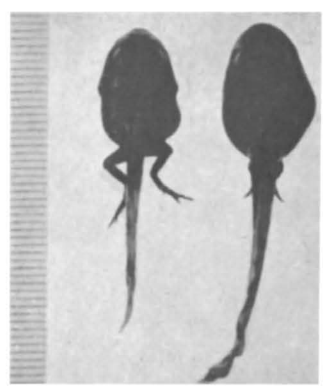

Control
Triploides

Control (X) animals and colchicin animals at different stages of development.

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