Experimental Studies of Abnormal Nuclear and Cell Divisions

II. Observation with living cells of the effects of alkalies and acids

By

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The direct effects of alkalies and acids on chromosomes and nuclei have been studied by many investigators. KUWADA and SAKAMURA (1927), SAKAMURA (1927), and YAMAHA and ISHII (1932) have made contributions to the relation between the H-ion concentration of mounting media and the visibility of chromosomes. The indirect effects of alkalies on nuclei have been studied by SINKE (1939) (with ammonia) in the young petal cells of Tradescantia reflexa and the guard cells of stomata in Tradescantia virginica, and those on mitosis by many other authors:—In animal cells KORNFELD (1926) has observed the effect of an acid and alkali, and ROSENFELD (1933) the effect of ammonia; in plant cells YAMAHA (1927) has studied the effect of acids and alkalies, and MAINX (1924) the effect of ammonia; they all used the fixation method. Recently, WADA has observed the effect on mitosis of ammonia vapour (1937) and acetic acid vapour (1939) with living hair cells of Tradescantia reflexa.

In the present investigation, the effects of alkalies and acids on mitosis were studied with living cells of leaf epidermis and young petals of Tradescantia reflexa, the same materials as those that I had used in the investigation of the effects of neutral salts and heavy metal salts (Sigenaga, 1944).

In this investigation Merck's preparations were exclusively used. They are as follows:—

Alkalies: ammonia and sodium hydroxide.
Salts of alkaline reaction: sodium bicarbonate and secondary sodium phosphate.
Acids: acetic acid and lactic acid.

Observation

1) Effects of alkalies

i) Effects of ammonia. Material: leaf cells and petal cells. Concentrations lower: M/10, M/50, M/100, M/200, M/400, M/1000, M/5000
and M/10000. Concentrations higher: 1%, 2%, 4%, 7.5% and 30%.

The effects of ammonia can be divided into two cases, the case of lower concentrations and that of higher concentrations. Generally speaking, the effect of dilute solutions can induce the formation of di-diploid nuclei or bi-nucleate cells. Under the influence of a M/10 solution, a solution of low concentration, the chromosomes are clumped together into a homogeneous mass and the spindle is contracted, with an associated phenomenon of enlargement of cytoplasmic vacuoles with granules in Brownian movement within, which may occasionally give rise to the formation of a pair of large vacuoles occupying the polar regions. The resting nuclei in the cells in the neighbourhood of the dividing cells also decrease in volume, and the chromonemata which are more or less indistinct are thicker in diameter and appear to be less in number, showing a coarser structure as a whole than in the untreated nuclei. When in this condition the medium is replaced with water, the vacuoles in the cytoplasm become much larger, and the spindle is rendered still narrower and smaller. The chromosome masses and nuclei also decrease still further in volume, finally become small, highly refractive masses of homogeneous structure, and the cells are brought to death in the end. In other instances, on the other hand, the vacuoles in the cytoplasm are much more enlarged on the replacement of the medium with water, but later the normal condition is restored. The spindle and the chromosome mass also decrease still further in volume, but in the latter a hydration follows then, while in the former the original condition can no longer be restored. In this case the final result is the formation of di-diploid nuclei or bi-nucleate cells. When the effect is weaker the spindle can be restored to a certain extent after the replacement of the medium with water, and a rudimentary cell plate is formed, thus resulting in the formation of bi-nucleate cells with a half made septum membrane. The following examples from protocol will give some details of process of these changes.

a) The case where a di-diploid nucleus was produced.

At 2.15 (p.m.). Material (leaf cells) was mounted with a M/10 NH₄OH solution, and a cell in meta-anaphase was subjected to continued observation under the microscope. The vacuoles in the cytoplasm was somewhat enlarged, but the chromosomes were nearly normal.

At 2.20. Granules in Brownian movement appeared in the enlarged vacuoles. The spindle decreased in bulk and the chromosomes were clumped together into homogeneous mass. Resting nuclei in the neighbourhood of the cell under observation decreased in volume, and the chromonemata within became thicker in diameter and less in number, and were only obscurely visible. Here the medium was replaced with water.

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1 A 3.5% solution approximately corresponds with the 1M solution.
At 2.30. The vacuoles in the cytoplasm were more strongly enlarged and each polar region of the cell was occupied by a large vacuole in which the granules in Brownian movement were found. The chromosomes which were individually distinguishable were found arranged in the form of a band in side view and confined in the equatorial region of the cell between the large polar vacuoles. In the cells in resting stage too, the vacuoles were found enlarged and each cell was occupied by a huge central vacuole, with the nucleus displaced to a corner of the cell. The nuclei in these cells were somewhat larger in this period and showed the chromonemata clearer. From this period on, the cytoplasm gradually showed the tendency to recover the normal condition.

At 4.00. The chromosomes clumped together formed a round mass (in side view), in which individual chromosomes were clearly observable. The vacuoles in the cytoplasm were now smaller, and the cytoplasm was returning to the normal condition.

At 4.15. Do. The chromosomes became somewhat obscurely visible.

At 5.35. The chromosomes in one group began to undergo the nuclear reconstruction process. The cytoplasm was now nearly normal. The resting nuclei were also found to be in the normal condition.

At 6.00. The nuclear reconstruction process was almost complete, and a di-diploid nucleus was formed, the spindle having disappeared.

b) The case where neither di-diploid nucleus nor bi-nucleate cell was formed.

At 11.30 (a.m.). Observation was commenced with material (leaf cells) mounted with city water. The cell under observation was in meta-phase.

At 11.36. The mitosis was in meta-anaphase. Here the medium was replaced with a M/10 NH₄OH solution.

At 11.40. Large vacuoles appeared in both polar regions of the cell. The spindle decreased in volume and the chromosomes were clumped together into a homogeneous mass.

At 11.42. Do. The solution was replaced with water.

At 12.00. The vacuoles grew larger, and the chromosomes which were obscurely visible were found occupying, in the form of a band (side view), a narrow space in the equatorial region of the cell between the large vacuoles. In the resting cells, the nuclei were pushed to a corner of the cell. In these nuclei the chromonemata were clearly seen.

At 4.00 (p.m.). The chromosomes were a small, refractive mass of homogeneous structure. In this case the cytoplasm did not return to the normal condition, and was found highly refractive, lining the cell wall and surrounding the periphery of the chromosome mass. The resting nuclei also became small refractive masses of homogeneous structure.
c) The case where a di-diploid nucleus and a bi-nucleate cell each with a half made septum membrane were formed.

At 1.30 (p.m.). Observation was commenced with material (leaf cell) with two anaphase cells, mounted with a M/10 NH₄OH solution.

At 1.35. With the enlargement of the vacuoles, the spindle decreased in volume and the chromosomes became a homogeneous mass. The resting nuclei also decreased in volume, and the chromonemata within increased in thickness and were somewhat obscurely visible. Here the medium was replaced with a M/1000 acetic acid solution.

At 1.45. The vacuoles grew much larger and the chromosome mass was pushed to one corner of the cell. The chromosome mass was smaller, but individual chromosomes became visible again. The resting nuclei were also displaced to a corner of the cells as a result of the enlargement of the vacuoles. The chromonemata were clearly visible in these nuclei.

At 1.55. The chromosome mass increased in volume, and individual chromosomes were somewhat obscurely visible. The resting nuclei also increased in volume, and the chromonemata decreased in thickness and refractivity. Here the solution was replaced with water.

At 3.00. The cytoplasm returned nearly to the normal state. In one (a) of the two cells under special observation, the daughter chromosome groups in anaphase were slightly apart from each other and in the other cell (b) the long axes of the two chromosome masses were in contact with each other with their ends and turned about 60°. The resting nuclei were almost normal.

At 5.20. The chromosomes began to undergo the nuclear reconstruction process. The phragmoplast swelled slightly, and in the cell (a), the cell plate was being formed between the two daughter chromosome groups. In the cell (b), daughter chromosome groups were fused together, forming a di-diploid nucleus of a dumb-bell shape. On one side of this compound, di-diploid nucleus, an incomplete cell plate was formed close to it, making an angle, about 30°, with its long axis.

At 9.00 (next morning). The nuclear reconstruction was complete in both these cells, while the formation of the septum membrane was incomplete. In the one cell (a) in which two independent nuclei were formed a half made rudimentary septum membrane of an L shape was observed between the nuclei, and in the other cell (b) in which a compound nucleus was formed a rudimentary septum membrane was found with nearly the same angle to the longer axis of the nucleus as that observed at 5.20 p.m. in the preceding day.

In this third example the medium (M/10 NH₄OH) is replaced with a M/1000 acetic acid. According to SINKE (1939), the nuclei which are dehydrated by a NH₄OH solution are brought again to swelling, when
the solution is replaced by a dilute acetic acid solution (M/10000). It appears that dilute acetic acid is a more favourable agent than water to remove the influence of NH₄OH. In this series of experiment, on the other hand, we often met with the case, where the replacement of the NH₄OH medium with a M/10000 acetic acid solution could cause the dehydrated chromosomes in anaphase neither to migrate to poles nor to undergo the nuclear reconstruction process, but resulted in the change to transform the chromosome groups into highly refractive masses. And such was often also the case with being used water instead of the acetic acid. The case of the third example may be regarded as an intermediate case between those in which the replacement of the NH₄OH medium with acetic acid can give rise to a normal progression of the mitotic process and in which it cannot, but a suspension of the process may occur. The spindle would in this case not have been so strongly dehydrated as it was entirely denaturalized, hence it would not have completely lost its recovering power. It could be restored in a certain degree and could form a half made septum membrane, when the replacement of the medium was made with acetic acid before the action of NH₄OH was too strong.

The effect of a M/50 NH₄OH solution was studied only in a few cases, with petal cells which were in metaphase. In this concentration also the vacuoles are enlarged, and the chromosomes are clumped together in a mass, but in a less strongly dehydrated condition than in the case of the M/10 solution in which the individual chromosomes are only obscurely observable. The resting nucleus also presents such dehydration phenomena as the decrease in volume and the increase in diameter and the apparent decrease in number, of the chromonemata. After the replacement of the solution with water a further enlargement of the vacuoles in the cytoplasm and the decrease in volume of the chromosome mass occur as in the case of the M/10 solution, but in a lower degree. From these changes the

Figs. 1—4, enlargee photomicrographs, originally taken with a Leica camera (Leitz) using Zeiss' oil immersion 1/12 and Leitz's peripl. oc. ×10. Figs. 5 a and b are those taken with a Makam (Leitz) using Zeiss' oil immersion 1/12 and Leitz's peripl. oc. × 8, and reproduced in the original magnification. Fig. 1 (a—e), showing di-diploid nucleus formation under the influence of M/100 NH₄OH solution. a and b, in the solution; c—e, after the medium replacement with water. Fig. 2 (a—c'), showing the same by fusion of two telophase chromosome groups under the influence of M/100 NH₄OH solution. a and b, in the solution; c, after the medium replacement with water. Fig. 3 (a—d), showing di-diploid nucleus formation by M/100 NaOH solution. a and b, in the solution; c and d, after the medium replacement with water. Fig. 4 (a—c), showing bi-nucleate cell formation by 1% sodium bicarbonate solution. a and b, in the solution; c, after the medium replacement with water. Fig. 5 (a and b), the case of di-diploid nucleus formation by fusion of two anaphasic chromosome groups under the influence of M/25 acetic acid solution. a, showing one in earlier stage than one in b, both in water medium.
normal or original states are soon recovered, however, and the mitosis continues to proceed with some delay in the rate of its progress. As mentioned in the case of neutral salts (Sigenaga, 1944), the formation of di-diploid nucleus from the chromosomes at metaphase is again difficult and it is true of all the other cases of experiment with lower concentrations. In the M/50 concentration, the di-diploid nuclei or bi-nucleate cells are easily obtainable only with the cells at meta-anaphase, anaphase or telophase.

Experiments with a M/100 NH₄OH solution were made with both petal cells and leaf cells. In this concentration too, the vacuoles in the cytoplasm are gradually enlarged, finally to push the cytoplasm to the peripheral regions of the cell and the spindle area, leaving only a few refractive cytoplasmic strands to stand as they are, that connect between these two regions without showing any streaming nor Brownian movement of granules. The resting nuclei are, as reported by Sinke, rendered small and highly refractive, with their chromonemata appearing thicker in diameter and less in number, being clumped together into a mass. The chromosomes in meta-anaphase can not migrate to poles (Figs. 1b and c). The two anaphasic chromosome groups come to approach each other to form one mass. The two telophasic chromosome groups also approach and come in contact with each other, and the initial, incomplete cell plate disappears (Figs. 2a and b). After the replacement of the solution with water, the nuclear reconstruction process takes place gradually, while the spindle (phragmoplast) becomes denaturalized, and the bi-nucleate cells or di-diploid nuclei are formed (Figs. 1e and 2c). In general, bi-nucleate cells are formed when the cells in anaphase are treated, and di-diploid nuclei are produced when the cells in late metaphase or in meta-anaphase are subjected to the treatment. In some cases, however, di-diploid nuclei are formed from anaphase or telophase chromosome groups by the fusion of the two groups being drawn together (Figs. 2a–c).

It was also observed that prophase nuclei too, can undergo the nuclear reconstruction process when the solution is replaced with water.

A M 200 NH₄OH solution can also show an effect, but much weaker one. In the case of petal cells, the mitosis at metaphase can progress normally to the end. It seems expectable that a prolonged treatment commenced at an earlier stage might cause an abnormal mitosis (the formation of di-diploid nucleus or bi-nucleate cell). In the case of leaf cells, on the other hand, di-diploid nuclei are often formed. Bi-nucleate cells with an incomplete septum membrane such as those mentioned in the case of the M/10 NH₄OH solution are also obtained. However, a di-diploid nucleus or a bi-nucleate cell completely devoid of the septum membrane formation is rather difficult to obtain even in this case. In
certain cases the mitotic process continues normally to the end, after the solution is replaced with water.

In a M/400 NH₄OH solution, in most cases, both di-diploid nucleus and bi-nucleate cell are difficult to obtain with leaf cells as material, and in a few cases it is possible to obtain bi-nucleate cells with a half made septum membrane only, which formation is due to some difficulty of the spindle to recover when the solution is replaced with water. In the majority of cases the spindle can swell back to the normal state, and the mitosis continues to proceed normally up to the end. In one case di-diploid nucleus was formed when the treatment with a M/400 solution was made two times. In this case, the replacement with water of the solution after the first treatment caused the mitosis to proceed further on, i.e., from early anaphase to late anaphase. In the second treatment with the solution, the dehydration of the spindle and chromosomes was brought about in a sufficient degree and the chromosomes were clumped together into a mass to result in the formation of a di-diploid nucleus after the second replacement of the medium was made with water.

In a M/1000 solution, most of the nuclear divisions proceed normally, and normal daughter cells are formed without the replacement of the solution with water.

In M/5000 and M/10000 solutions no abnormalities in division are observed. All mitoses proceed normally.

The effects of higher concentrations than 1% were studied with petal cells. They are similar in both cases of 1% and 2% solutions. Immediately after the mounting medium of water is replaced with a 1% or a 2% NH₄OH solution, the vacuoles in the cytoplasm are enlarged, granules becoming visible in them. In the resting nucleus the chromonemata grow thicker and appear to be less in number. They are only more or less obscurely visible, and soon come to disappear from sight, the whole structure of the nucleus being rendered homogeneous. In metaphase and anaphase also, individual chromosomes become invisible forming a homogeneous mass, and a large vacuole is found in each polar region of the cell. In telophase, another large vacuole comes out to occupy the central cell region, the region of the phragmoplast. These vacuoles grow larger and larger, while the nucleus or the chromosome mass or masses are made smaller in an inverse proportion. Finally, almost the whole cell cavity becomes occupied with large vacuoles which push the cytoplasm to the cell wall and the nucleus or the chromosome mass or masses to make it or them assume the form of a thin band with high refractivity. Then, the cytoplasm, the chromosomes and the nucleus, all come to swell, and the whole cell appears almost hyaline. When these swollen chromosomes are fixed and stained with acetocarmine, they show a karyomere
structure with their chromonemata very much loosely coiled. The resting nucleus is gradually contracted when stained, and first granules, and then chromonemata, become visible in it. Sometimes it shows a mesh-like structure.

In 4%, 6% and 7.5% solutions the vacuoles in the cytoplasm are enlarged to a certain degree, and the chromosomes and the nucleus become dehydrated into a homogeneous mass, just in the same way as observed in the first stage of affection by 1% and 2% ammonia solutions. In the solutions of concentration here in question, however, the chromosomes and the nucleus become swollen again from this stage of dehydration, and do not show such a great decrease in volume as observed in the latter case. The nucleus swells finally to such an extent that it almost occupies the whole cell cavity, with granules which appear on its swelling in it and with its boundary which is still recognizable. The chromosomes also swell with their recognizable boundary. These changes are shown more remarkably in stronger solutions. In a 30% solution the nucleus swells with granules clearly visible in it, and the chromosomes present the figure of the so-called chromosome negative. When these cells are stained with aceticarmine, the nucleus shows a coarser chromonema structure or sometimes a mesh-like structure, and the chromosomes also the structure composed of chromonemata loosely coiled. The fixed prophase nucleus shows the structure which hardly differs from that of the resting nucleus. In these experiments with the solutions of higher concentrations, no experiment of replacing the solutions with water was made, but it is suggested that di-diploid nuclei or bi-nucleate cells may be obtained if the medium is replaced with water at an early stage of affection, due to the fact that in these solutions of higher concentrations, at least those up to 7.5%, dehydration figures are observed in the first stage of affection.

ii) Effects of sodium hydroxide. Material: petal cells and leaf cells. Concentrations: M/50, M/80, M/100, M/120, M/200, M/500, and M/1000.

In a M/50 solution the vacuoles in the cytoplasm are enlarged as extensively as observed in the case of the 1% or 2% ammonia solution, and the chromosomes in metaphase are transformed into a homogeneous mass of the form of a narrow band (in side view) and are located in the middle region of the cell between the large vacuoles in polar regions. This chromosome mass soon swells to occupy the whole cell interior, and when it is stained with aceticarmine, it shows a structure like a resting nucleus, and is found surrounded by a hyaline area.

In a M/80 solution, the chromosomes and the nuclei become swollen when a longer treatment is made. In a shorter treatment, the vacuoles in the cytoplasm are enlarged to a certain extent, but not so strongly as
in the case of the M/50 solution. The chromosomes and the spindle are dehydrated, and the former are transformed into a mass (in metaphase) or masses (in anaphase). When the solution is replaced with water at this stage of change, the vacuoles in the cytoplasm are enlarged still further, and the chromosome masses become coagulated to small, highly refractive, homogeneous masses. In the case of petal cells the influence is weaker. The chromosomes are clumped together, and become obscurely visible, but on the replacement of the solution with water, the chromosome mass comes to show a chromonema structure similar to the resting nucleus, and then swells into hyaline appearance. The resting nuclei also swell after the replacement of the solution with water, and become hyaline except for nucleoli and sometimes also granules which both are now visible.

In a M/100 solution, the chromosomes and the spindle are sufficiently dehydrated (Figs. 3a and b) resulting, when cells in metaphase or early anaphase are treated, in the formation of di-diploid nuclei (Fig. 3d), and the manner of affection in this case is comparable to the case of the M/100 NH$_4$OH solution. Bi-nucleate cells are also formed from the cells in anaphase and telophase just in the same manner as in the case of the cells treated with the M/100 NH$_4$OH solution. In the metaphase, the chromosomes are in the clumped state, but when the solution is replaced with water anaphase can follow and the mitosis is normally completed. In this concentration, the influence upon producing chromosome clumping is often stronger than in the M/100 NH$_4$OH solution, as especially seen in the experiments with leaf cells, and the chromosomes and the spindle become dehydrated more and more strongly even after the replacement of the solution with water is made, resulting in the cases of metaphase and anaphase in a very small, highly refractive, homogeneous mass or masses of chromosomes. The resting nuclei also decrease in volume in this solution, and the chromonemata increase in thickness and refractivity. After replacing the solution with water, the chromonemata come back to the state in the normal resting nuclei. In many cases, however, when the medium replacement is made, the nuclei become either contracted into small, homogeneous bodies or swollen into large hyaline bodies containing visible nucleoli and granules within.

In a M/120 solution, the effects are the same ones as in the case of the concentration, M/100, and bi-nucleate cells and di-diploid nuclei are formed.

In a M/200 solution, sometimes the di-diploid nuclei and sometimes also the bi-nucleate cells are formed, but sometimes the mitosis (at prophase) can proceed normally to the end in the solution, without receiving the medium replacement with water.

In M/500 and M/1000 solutions the mitoses proceed quite normally.
2) Effects of salts of alkaline reaction

i) Effects of sodium bicarbonate. Material: petal cells and leaf cells. Concentrations: 0.1M and 0.2M in the case of petal cells and 1%, 1.5%, and 2% \(^1\) in the case of leaf cells.

Generally speaking, di-diploid nuclei and bi-nucleate cells (Fig. 4c) are obtainable in both cases of material. The manner of affection is similar in all these cases of different concentrations to the case of M/100 ammonia solution (Figs. 4a and b), though in some cases, bi-nucleate cells with an incomplete septum membrane are formed. In general, when the cells are affected strongly, the dehydration effect on chromosomes and nuclei is strong, and the dehydrated state remains unchanged even after the replacement of the solution with water, they being small, refractive, homogeneous masses. This phenomenon is more frequently the case with leaf cells than with petal cells. In some other cases the chromosomes and nuclei swell to a hyaline mass, and in the case of resting nuclei only nucleoli and granules are visible structures. It is highly probable that these hydration changes are direct effects of the solution on the nuclei and chromosomes, and that in these cases the cells had sustained an injury and lost their semipermeability before they were subjected to the treatment with the solutions.


Concentrations: 0.2M, 0.15M, 0.1M and 0.05M.

In a 0.2M solution, the cells are plasmolyzed, and the nuclei and chromosomes are brought to dehydrated condition. The chromosomes are clumped together to a homogeneous mass, and the nuclei decrease in volume with their chromonemata somewhat obscurely visible and appearing thicker in diameter and less in number. Soon the cells become deplasmolyzed, and the chromosomes and the nuclei come to swell into hyaline masses. When the solution is replaced with water, the chromosomes and the nuclei decrease in volume, and the vacuoles in the cytoplasm become enlarged. Finally the cells are brought to death with the nucleus or chromosome mass which is small in size and homogeneous and refractive. In metaphase this mass of chromosomes is sometimes uneven in contour and in anaphase it assumes a dumb-bell shape. When these nuclei and chromosome masses are stained with acetocarmine in swollen and hyaline appearing condition, the former come to present a coarse mesh-like structure and the latter a structure that is very similar to the former.

A 0.15M solution is also slightly hypertonic, and in this solution the cytoplasm is detached slightly from the cell membrane. As in the case of the 0.2M solution, the chromosomes and nuclei first show various

\(^1\) 1% = ca 0.12 M; 1.5% = ca 0.18 M; 2% = ca 0.24 M.
dehydration figures, but soon the cells become deplasmolyzed and the chromosomes and nuclei come to swell.

In a 0.1M solution, plasmolysis no longer occurs, but the vacuoles in the cytoplasm become enlarged, and the individual chromosomes are rendered obscure, being clumped together into a mass. When the solution is replaced with water at this stage of the change, the individual chromosomes become visible somewhat clearly, and then gradually begin to over-swell and undergo the nuclear reconstruction process, resulting in the formation of a di-diploid nucleus or a bi-nucleate cell. These abnormal nuclei or cells are formed when cells at metaphase or late prophase are treated, and not when they are at anaphase. This is chiefly due to the fact that in this solution the dehydration of the chromosomes and spindle is a tardy process, and it takes much more time to suspend the mitosis completely than in the other solutions. In anaphase and also in late metaphase the mitosis often proceeds to telophase, and after the replacement of the solution with water the two chromosome groups form nuclei with a septum membrane between them. The mitoses in early and middle prophase can proceed to late prophase, and these nuclei are transformed at this stage directly into resting nuclei when the solution is replaced with water. In some cases, the mitosis in middle prophase can complete its whole process when the medium replacement with water is made at late prophase.

In a 0.05M solution the vacuoles in the cytoplasm are gradually enlarged in some degree, and the resting nucleus is rendered somewhat more refractive. But the mitosis proceeds normally from metaphase and also from anaphase on to the end, and hence neither di-diploid nuclei nor bi-nucleate cells can be obtained from the treatment of the cells in these mitotic stages.

**iii) Effects of McIlvaine's buffer solution in pH 7.0.** The buffer solution consists of 16.47 cc. of 0.2M Na₂HPO₄ and 3.53 cc. of 0.1M citric acid. In this solution petal cells are plasmolyzed, and the chromosomes in metaphase and anaphase are caused to be a homogeneous mass or masses. When the solution is replaced with water, the cells are deplasmolyzed and the vacuoles in the cytoplasm are enlarged to a certain extent, and the chromosomes are rendered again visible. The cytoplasm soon comes to recover its normal condition, and the chromosomes swell and gradually undergo the nuclear reconstruction process, while no septum membrane is formed. Thus the result is the formation of a di-diploid nucleus or a bi-nucleate cell. In some cases, no plasmolysis occurs, but the chromosomes and spindle are dehydrated along with the gradual enlargement of the vacuoles in the cytoplasm. In these cases too, after the replacement of the solution with water a di-diploid nucleus or a bi-nucleate cell is formed.
While in the first case the formation of the di-diploid nucleus and bineucleate cell is regarded chiefly as an effect of hypertonicity of the solution, in the second case it is very likely an indirect effect of Na₂HPO₄. In this second case no plasmolysis occurs, hence it may be considered that the cell is somewhat different from the normal one in the property of permeability owing to some unfavourable environmental condition, or to an injurious effect on the cell of detachment of the petal from the bud, to permit in some measure the penetration of the solution into the cell. In the first case too, a longer treatment with this solution caused deplasmolysis, and the chromosomes and nuclei gradually swell to hyaline masses with an increase in volume.

3) Effects of acids

i) Effects of acetic acid. Material: petal cells. Concentrations: M/10, M/25, M/50, M/100, M/200, M/400, M/1000 and M/10000.

In these solutions the effect of acid is always a direct one. The chromosomes and chromonemata increase in refractivity and finally become coagulated, showing similar coagulation figures to those that are commonly observed in fixed or acetocarmine preparations. This coagulation first sets in after some progress of mitosis. For instance, the anaphase chromosomes can migrate to poles, but here they are coagulated. In this case fibrous structure appears in the phragmoplast. When the medium is replaced with water at this first stage of coagulation, the cells can in some cases recover the normal condition, and the mitosis continues to proceed to the end, some fibrous aspect being recognizable for a while in the phragmoplast. In other cases, on the other hand, the chromosomes and spindle become dehydrated as the vacuoles in the cytoplasm are enlarged, and the chromosomes are transformed into highly refractive, homogeneous mass or masses. In anaphase and telophase, the achromatic figure remains as a somewhat refractive mass of compact texture. The resting nucleus becomes also smaller and shows a homogeneous or a coarse mesh-like structure. In one case (M/25), it was found that the vacuoles in the cytoplasm were enlarged in the first moment after the replacement of the medium with water, and the two anaphase chromosome groups came in contact to fuse each other and underwent the nuclear reconstruction process, while the phragmoplast was denaturalized (Fig. 5a). In this case the result was the formation of a di-diploid nucleus (Fig. 5b).

Generally speaking, the more the acid is dilute, the further the mitosis can proceed on before coagulation sets in. The coagulation of the protoplast in the solution and the enlargement of vacuoles in the cytoplasm that occurs after the replacement of the medium with water, however, are common to almost all the cases treated with the acetic acid.
solutions in concentrations from M/10 to M/100, and generally under the influence of acetic acid, the formation of a di-diploid nucleus or a bi-nucleate cell is extremely difficult; this is due either to the fact that the coagulation generally proceeds further even after replacing the medium with water, or to the fact that the natural condition of the cell becomes in most cases completely restored on the medium replacement, further to continue the mitosis. Out of the 50 cells observed, only one cell showed the di-diploid nucleus formation.

In M/200 and M/400 solutions, the vacuolization takes place in the cytoplasm, and the resting nucleus decreases in volume, showing a hyaline area in the circumference of the nucleolus. The chromonemata become thicker and are sometimes coagulated into a mesh-like structure as a whole. The chromosomes are also coagulated and are somewhat clumped together. The vacuolization in the cytoplasm and the diminution in size of the resting nucleus seem to be stronger in the M/400 solution than in the M/200 solution. In the case of these solutions too, neither di-diploid nuclei nor bi-nucleate cells are brought forth, but the mitoses can continue to proceed normally if the replacement of the solution with water is made.

In the solutions of the concentrations, M/1000 and M/10000, the mitoses proceed quite normally from late prophase to the end, showing no sign of coagulation. In some cases, however, the spindle which becomes more or less contracted is somewhat difficult to swell or to spread out again, and the mitotic axis is turned round in some degree, the septum membrane accordingly being formed obliquely.

**ii) Effects of lactic acid.** Material: petal cells. Concentrations: M/10, M/25, M/50 and M/100.

The results show that the effects of these acid solutions on the same material are the same as those of the acetic acid solutions; the mitoses in progress stop and the cells become coagulated. When, however, the solutions are replaced with water at the first stage of coagulation, the vacuoles in the cytoplasm are enlarged and the chromosomes and the spindle are dehydrated, and then, the spindle being excepted, the chromosomes and the cytoplasm are brought to the normal or a more hydrated condition. The chromosomes thus undergo the nuclear reconstruction process, while the formation of septum membrane is suspended, and the final result is the formation of di-diploid nuclei or bi-nucleate cells. In the present investigation, in a M/10 solution the formations of a di-diploid nucleus and a bi-nucleate cell were observed each in two cases. In one case a di-diploid nucleus which appeared hyaline as a result of an excessive hydration was formed. This nucleus showed the chromonema structure when it was stained with acetocarmine. In other cases, however, even
after the replacement of the medium with water, the spindle and chromosomes are more and more dehydrated, accompanied with a further enlargement of the vacuoles in the cytoplasm, finally resulting in the formation of a small, highly refractive, homogeneous mass. And in some others the cells are brought back to the normal condition, and the mitoses continue to proceed normally to the end. Bi-nucleate cells with an incomplete septum membrane as well as those cells are also formed, in the latter of which an incomplete septum membrane extending from one side of the cell wall to one of the nuclei is found in the orientation parallel to the long axis of the cell. The latter case may be regarded as due to turning round of the mitotic axis in some degree. A similar case was also observed under the influence of 0.15M KNO₃ solution, as mentioned in the previous paper (Sigenaga, 1944).

In M/25, M/50 and M/100 solutions also, the cells are coagulated. In these cases a replacement of the medium with water made at the first stage of coagulation does not result in the formation of di-diploid nuclei or bi-nucleate cells, but in two cases only, a bi-nucleate cell with a trace of an incomplete septum membrane was obtained. In these experiments a change in orientation of the axis of mitotic figures was often observed. In some cases, the phragmoplast was recovered from dehydration after the replacement of the medium with water, and the septum membrane was formed first on one side only and then on the other too. It is to be added here that the telophasic nuclear reconstruction process may sometimes take place earlier in one of the daughter chromosome groups than in the other.

Conclusion

The results we obtained show that alkaline solutions and acids can induce in proper concentrations the formation of di-diploid nuclei and bi-nucleate cells, provided the treatment is followed by the replacement of the media with water. Though the manner of affection differs in detail according to alkalies or acids used, the main process of the changes due to affection is the same in both these cases, being first the dehydration of the spindle or phragmoplast and chromosomes, and then an untimely occurrence at any stage of mitosis of the nuclear reconstruction process not accompanied by the septum membrane formation after the replacement of the media.

Generally, in the case of alkaline solutions of high concentrations swelling occurs in chromosomes and nuclei, and only when fixed, the internal structure becomes visible in both these structures. This swelling is the direct effect of alkalies on chromosomes and nuclei, and in the case of chromosomes this result is the same as when the cells which have
lost their semipermeability are treated with a dilute alkaline solution—the artificial chromosome unravelling (Kuwada and Nakamura, 1934; Sinke and Oura published with Kuwada, 1938). The present author was once of the opinion that the alkaline solutions can affect chromosomes and nuclei only directly, and the di-diploid nuclei and bi-nucleate cells which are observed after fixation of the material are merely fixation figures at metaphase, anaphase or telophase. From this point of view, in the previous paper (1937) it was assumed that the results obtained by Mainx (1924) and Rosenfeld (1933) under the influence of ammonia were such fixation figures of swollen chromosomes. But, Rosenfeld has observed the clumping together into one group of chromosomes in the living state of the cell, and hence it is highly probable that in this case the chromosome clumping is a phenomenon due to the dehydration of the chromosomes and spindle as an indirect effect of the ammonia buffer solution or ammonia vapour which he used in his experiments. Kornfeld (1925) has also observed that in aster stage the chromosomes clump together, and in di-aster stage the chromosome bridge is formed, as a temporary effect of KOH solution on cornea cells of urodelian larva. These indirect effects are comparable to the cases of alkaloids, narcotics and neutral salts from the physiological point of view.

In his investigation with ammonia, Rosenfeld has also obtained the result that the reconstruction of the nucleus from the clumped chromosomes takes place when the affecting medium is replaced with the culture medium. Mainx (1924) has obtained bi-nucleate cells in root-tip cells of Zea mays and Vicia Faba by treating them in a 0.1M NH₄OH solution. He states that ammonia shows a similar effect to those of chloralhydrate, chloroform and alkaloids, though it is liable to bring the cells to death. He is of the opinion that the principal effect of ammonia on mitosis is the swelling of the spindle. Yamaaha (1927) has also observed the chromosome clumping and the anaphase bridge by the treatment with NH₄OH and KOH, and obtained the bi-nucleate cells with NaHCO₃ and Na₂CO₃. Rosenfeld, who has obtained the di-diploid nuclei and bi-nucleate cells as the effect of ammonia buffer solutions, has failed to obtain them with other alkaline buffer solutions such as borate-NaOH, glycolcoll-NaOH and Na₂CO₃-HCl. From these results he obtained he assumes that ammonia should have a special effect on mitosis. Barth (1929) has found that in Arhacia egg ammonia decreases the viscosity of the protoplasm, when the solution is lower than 9.6 in pH value, while it increases when it is of the pH value above this, and that at pH 10.0 coagulation occurs. Barth has also shown that NaOH causes no increase in viscosity in all the pH values (pH 8–11) he examined. He explains the increase in viscosity of protoplasm by the ammonia solutions of certain pH values as due to a
certain property of ammonia such as fat solving or surface tension lowering action, rather than to OH-ion.

According to WADA (1937), in living staminate hair cells of *Trades-cantia reflexa* the chromosomes and cytoplasm are first swollen as the effect of ammonia vapour, and then undergo an "Entmischung" process, and finally the cells are brought to death by coagulation. LEPESHCHKIN (1923, cf. also 1938, p. 218) has also shown that when a *Spirogyra* plant is placed in a 2% sodium carbonate solution, the protoplasmic streaming becomes suspended and the nucleus is shrivelled, and then follow a strong granulation in both protoplasm and cell sap and the death of the cells. The coagulation or the increase in viscosity of protoplasm which follows the treatments with weak or dilute alkalies has also been observed by PRAT (1926)\(^1\), DARWIN (1882)\(^2\) and BOKorny (1888).

In the present investigation, the effects of NH\(_4\)OH, NaOH, Na-bicarbonate, and Na-phosphate, and also of McIlvaine's buffer solution of pH 7.0 were examined, and bi-nucleate cell and di-diploid nucleus were obtained as the final result. Since McIlvaine's buffer solution of pH 7.0 consists of 16.47 cc. of 0.2M Na\(_2\)HPO\(_4\) and 3.53 cc. of 0.1M citric acid, and since the effects of this buffer solution and Na\(_2\)HPO\(_4\) (0.1M) are similar to each other, as was given above in the descriptive part, it may be assumed that in the case of the buffer solution too, the cells are affected chiefly by Na\(_2\)HPO\(_4\). In the buffer solution of this pH value, 7.0, the solution is slightly hypertonic, and hence it seems likely that the dehydration in the beginning is an osmotic effect and the further dehydration is an indirect effect of the salt due to its penetrability in some limited degree. This view is supported by the fact that in a longer immersion in the buffer solution, the chromosomes and nuclei being swollen, direct effects are also shown, a fact which indicates that the solution may gradually penetrate into the cell in a certain ample amount. In a single solution of Na\(_2\)HPO\(_4\) too, when it is hypertonic, the cells first undergo the osmotic influence and are plasmovled, but then deplasmol ved as the solution penetrates into the cells, showing the chromosome and nuclear swellings. In the case of NaHCO\(_3\) solution (0.2M or 2%), the direct effects are also sometimes observed, but in this case the effects seem to be due to a special injury which the cells may sustain during preparation.

The results obtained by KORN Feld, YAMAHA, and LEPESHCHKIN as well as those obtained in the present investigation suggest that the dehydration of the protoplasm takes place not only as the effect of NH\(_4\)OH, but also of other alkaline solutions, if the concentrations are adequate. The specificity of NH\(_4\)OH that has been assumed by ROSENFLED and BARTH may

\(^1\), \(^2\) Cited from HEILBRUNN (1928).
possibly be in a certain connection with a stronger penetrability and a weaker action on general protoplasm of ammonia. Too high concentration would of course bring about the direct effect. The view here put forward seems to be supported by the result obtained in the present investigation that the di-diploid nucleus and bi-nucleate cells are obtained with more liability with NH₄OH than with NaOH.

The present investigation shows that the effects coincide more in detail with those of alkaloids, narcotics (SIGENAGA, 1937) and neutral salts (SIGENAGA, 1944), in the case of alkaline solutions of adequate concentrations than in the case of acids. The principal effects which are in coincidence are: the enlargement of vacuoles in the cytoplasm, the decrease in volume of the spindle and the increase in refractivity of the chromosomes, those occurring in the solutions, and the hydration of chromosomes, the restoration of the normal aspect of the cytoplasm and the denaturalization of the spindle, those observed after the solution replacement with water. The di-diploid nucleus or bi-nucleate cell is formed so long as these latter changes can take place (when the solutions are replaced with water) except the change that occurs in the spindle. In the alkaline solutions, resting nuclei also decrease in volume, and the chromonemata within increase in thickness and refractivity and appear to be reduced in number. These changes taking place in the resting nuclei and the vacuolization occurring in the cytoplasm under the influence of NH₄OH solution have been thoroughly studied by SINKE (1939), and are taken as evidence of dehydration which should occur under that influence. SINKE states: “In alkaline media the vacuoles in the cell swell and absorb water from the surrounding cytoplasm and nuclei, so that the cytoplasmic streaming stops and the nuclei are dehydrated.” This explanation of the nuclear dehydration in alkaline media may also be applicable to our cases of the effects on mitosis. The dehydration that thus occurs as indirect effect must give rise to the formation of a di-diploid nucleus or a bi-nucleate cell when the dehydrating condition is removed by replacing these media with water, as we have already seen in the cases of hypertonic solutions and various neutral salts solutions.

The final effect of acids is so far as the present investigation is concerned always coagulation of the whole protoplast, which is a direct effect and differs from that which occurs as an indirect effect in the case of a prolonged treatment with alkalies. This result agrees with those obtained by SINKE and some others. It seems highly probable that the influence of acids on chromosomes and nuclei is also a direct one. In these cases of acids, the replacement with water exerts mostly no influence on the recovery of the protoplasts, in accordance with the view of LEPESCHKIN.
(1937). When, however, the replacement is made at some appropriately early stage of acid effect, the nuclear reconstruction process may take place. First the vacuoles in the cytoplasm are enlarged, giving rise to a further dehydration of the chromosomes and spindle, and then the cytoplasm gradually restores its normal condition, and the spindle disappears without showing any restoration. Then, in this case too, a di-diploid nucleus or a bi-nucleate cell is formed. Recently WADA (1939) has observed that under the influence of acetic vapour the spindle undergoes a change into gelatinization, and this phenomenon is followed by the formation of di-diploid nuclei and bi-nucleate cells. It is naturally an extremely difficult task to find out an appropriate stage of replacing the medium, and so it is very much difficult to obtain di-diploid nuclei or bi-nucleate cells under the influence of acids.

In his investigation of the effect of hydrochloric acid on mitosis KORNFELD (1925) has obtained the following abnormal cells and mitoses: giant cells with two or more nuclei, nuclear budding, amitotic nuclear division, chromosome clumping in aster stage, and chromosome bridge in diaster stage. He has noted that in this case of his experiment the disturbance in mitotic process is not so intense as in the cases of experiment with X rays carried out by POLITZER and ARBERTI. YAMADA (1927) has also studied the effects of various acids (inorganic and organic) on mitosis, and has observed such abnormalities as anaphase or telophase bridge, but no di-diploid nucleus or bi-nucleate cell, except in the cases of butyric acid and formic acid, in both of which bi-nucleate cells were obtained. In this result of YAMADA's also, the difficulty of obtaining di-diploid nuclei or bi-nucleate cells under the influence of acids is shown.

The conclusions which may be drawn at present are as follows:—Under the affection by alkalies, the cytoplasm becomes strongly vacuolized, while it itself becomes increased in viscosity and the spindle and chromosomes are dehydrated, probably as a result of the water being taken out by the enlarged vacuoles. These changes proceed gradually and finally become irreversible, first in the spindle. When the medium is replaced with water at an appropriate stage of the change, the cytoplasm comes back to the normal state, and the chromosomes become able to undergo the nuclear reconstruction process, while the spindle is lost to view. Bi-nucleate cells or di-diploid nuclei are thus formed as final result.

Generally speaking, the effects on cells of alkalies and salt solutions of alkaline reaction are similar to those of alkaloids and narcotics (SICENAGA, 1937) and of neutral salts already mentioned. Strong alkalies (NaOH) act on cells more strongly than weak alkalies (NH₄OH), and under the influence of strong alkalies the formation of the abnormal nuclei and cells is difficult. As in the cases of heavy metal salts, the range of
effective concentrations and the effective time lengths of immersion is regarded as also narrow in these cases.

Under the affection by acids, protoplasm coagulation is brought about generally. In case, however, the medium is replaced with water at a reversible stage of coagulation, the cytoplasm becomes strongly vacuolized, and while the spindle is denaturalized, the chromosomes gradually swell to form a di-diploid nucleus or a bi-nucleate cell. In this latter case, the denaturalization of the spindle and phragmoplast, the total suppression of mitosis by coagulation, and the untimely nuclear reconstruction process that occurs after the replacement of the medium with water, also take place as in the cases of alkaloids, narcotics and neutral salts, but the effective range of concentrations and effective time lengths of immersion is very narrow, and in this respect this case of acids is comparable to the case of heavy metal salts or the case of strong alkalies.

Summary

1) Ammonia solutions of low concentrations cause the dehydration of chromosomes and the spindle and also an associated phenomenon of the enlargement of vacuoles in the cytoplasm, and after the replacement of the solutions with water di-diploid nuclei and bi-nucleate cells are formed. The manner of affection is the same as in the cases of alkaloids, narcotics and neutral salts.

2) Sodium hydroxide affects mitosis in a similar manner to ammonia, but the ranges of effective concentrations and effective time lengths of immersion, in which range an adequate dehydration of chromosomes and the spindle is brought about and di-diploid nuclei or bi-nucleate cells can be formed after replacing the solution with water, seem to be narrower than in the case of ammonia. In this respect this case is comparable to the cases of heavy metal salts.

3) In salt solutions of alkaline reaction (Na₂HPO₄, NaHCO₃) in medium concentrations, chromosomes and the spindle are also dehydrated in association with the enlargement of the vacuoles in the cytoplasm, and di-diploid nuclei or bi-nucleate cells are formed when the replacement of the solution with water is made. The manner of affection is similar to the case of ammonia solutions of low concentrations.

4) Acids (acetic acid and lactic acid) always act on cells as coagulating agent. After the replacement of the acids with water, the vacuoles in the cytoplasm are enlarged temporarily, and chromosomes and the spindle are dehydrated, but in this case the formation of di-diploid nuclei and bi-nucleate cells is possible only when the medium is replaced with water at the first stage toward coagulation and is successful only in a few
cases. In this case the range of effective time length of immersion is very narrow as in the cases of heavy metal salts.

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